STUDIES ON THE MICROORGANISMS OF A FRESHWATER LAKE

A thesis

presented for the degree

of

Doctor of Philosophy in Microbiology

in the

University of Canterbury,

Christchurch, New Zealand.

by

Angela J. Ramsay

1972

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

No. Page

ABSTRACT

A study was made of the heterotrophic bacteria of Lake Grasmere, an oligotrophic to mildly eutrophic freshwater lake, situated at an altitude of about 600 m in inland Canterbury, New Zealand. The maximum depth of the lake is 12 m and there are beds of Elodea canadensis around most of the lake to a depth of about 6-7 m. During the period of study, between 1969 and 1971, the lake was well oxygenated and did not stratify.

The following parameters were found to be positively correlated:

a) numbers of bacteria and total organic nitrogen in samples from over the inflow of a spring, which entered the lake beneath 2 m of water;

b) numbers of bacteria and total organic nitrogen in the open water;

c) numbers of bacteria over the spring and rainfall in the week before sampling.

Correlation coefficients were not significant between:

a) numbers of bacteria and nitrate nitrogen over the spring;

b) numbers of bacteria in the open water and rainfall in the week before sampling;

c) numbers of bacteria in the water over Elodea and rainfall in the week before sampling.

However, the size of the bacterial population in the open water in the autumn appeared to be influenced by the amount of rainfall in the previous winter.

There was some increase in the numbers of bacteria in the water over Elodea in the autumn and winter months. This response was attributed to the presence of Elodea, but was not recorded during the rest of the year. The total numbers of bacteria on Elodea, and numbers of those metabolizing, as detected by autoradiographic experiments, increased as the leaves aged.

In the open water, numbers of bacteria increased a little after blooms of zooplankton, but there was little or no response to changes in phytoplankton populations, or to the presence of Elodea canadensis. Bacteria in the mud rarely increased in numbers after the deposition of organic matter.

The numbers of viable bacteria in the open water were outnumbered by algae by a factor of between 4 and 34. Few, if any, bacteria were detected on algae or zooplankton by cultural methods. No evidence was obtained that the epiphytic bacteria, observed on some algae, were involved in their decomposition.

The kinds of bacteria in the water varied considerably at different times of the year and these seasonal changes were also reflected to some extent in the microflora of samples of mud and Elodea. In the water, pseudomonads, Alcaligenes/Achromobacter, flavobacteria and coryneforms were usually the main groups of bacteria found, but Cytophaga, Vibrio extorquens, Enterobacteriaceae, Aeromonas/Vibrio and Micrococcaceae were abundant in some samples. Pseudomonads

and non-motile flavobacteria were predominant in three samples of Elodea characterized. Samples of concentrated plankton had larger proportions of flavobacteria, coryneforms and Vibrio extorquens, but smaller percentages of pseudomonads and Micrococcaceae, than the samples of untreated water. The microflora of a sample from the surface layer of mud was similar to the population in the water at that time, but mud from up to 8 cm below the surface had larger proportions of Gram-positive bacteria.

In the autumn of one year, isolates of Enterobacteriaceae, which were unlike Escherichia coli, were common in several areas of the lake. This was attributed to a combination of factors, including the presence of waterfowl on the lake. An association of Vibrio extorquens with products of plankton, and of Cytophaga with increased concentrations of certain unspecified minerals was suggested.

Leaching of nutrients essential for bacterial metabolism from organisms which had recently died, and competition with the primary producers for nutrients, were suggested as possible reasons for the frequent lack of reponse by bacteria in the water and mud to the addition of organic matter.

CHAPTER 1

INTRODUCTION

1.1 BACTERIA IN LAKES

Bacteria have frequently been neglected in limnological studies. Yet autotrophic bacteria may contribute significantly to the level of primary production in some lakes (Takahashi and Ichimura, 1968; Sorokin, 1970a), and heterotrophic bacteria are commonly assumed to be the main group of organisms responsible for the mineralization of organic matter (Waksman, 1941; Kuznetsov, 1968). In the present study only heterotrophic bacteria were considered. The anaerobic conditions favourable for such autotrophic bacteria as photosynthetic sulphur bacteria were not found in the lake investigated. It was always well oxygenated during the period of study.

Heterotrophic bacteria in lake water make up an extremely diverse group. They may be free-floating in the water or attached to surfaces such as detritus and plankton. Some may have originated from outside the lake and have been brought into the lake through inlets. Others may have been dislodged from the surfaces of organisms such as fish and macrophytes. If the lake is shallow and subject to wind action, bacteria from the mud may also be found in the lake water. With such a diverse population of bacteria there are difficulties in distinguishing between bacteria that have only a limited period of viability in the lake, and those that can grow and

proliferate in water.

The activity of bacteria in lake water must also be considered in relation to that of bacteria in other habitats within the lake. Bacterial degradation of organic matter in lake water may be insignificant compared to the amount of decomposition occurring in other sites such as on the surface of macrophytes or in mud. Nutrients may also be recycled by mechanisms other than bacterial action. Nutrients are released by autolysis and solution from organisms which have recently died, and zooplankton, when alive, have been found to excrete many times the amount of soluble nutrients available for recycling on their death (Johannes, 1968). Concentrations of nutrients may also be increased by inflow from sources outside the lake. All these other methods of maintaining nutrient levels in a lake may influence the activity of bacteria.

1.2 TERMINOLOGY

Organisms in different aquatic habitats have been described by a variety of terms. In the present study, the term 'planktonic' describes those organisms free-floating in the water and 'benthic' those inhabiting the mud (Potter, 1964). A third group of organisms attached to the surfaces of macrophytes or algae are termed 'epiphytic'. This term has been used to describe bacteria on the surface of Elodea canadensis (Strzelczyk, Antczak and Kuchcińska, 1971) and other macrophytes (Allen, 1971). With an aquatic plant, the surfaces of stems and roots, as well as leaves, are all suitable for colonization and a term which describes organisms attached to any part of the plant is preferable to a term,

such as 'phylloplane bacteria', which is commonly used to describe the bacterial flora of terrestrial leaves.

1.3 LITERATURE SURVEY

Within the field of aquatic bacteriology some studies have been confined to the bacteria of one habitat such as water or mud, while others have considered the relationship between heterotrophic bacteria and biological factors such as plankton. In some cases only counts of bacteria have been made, while in others bacteria have been isolated and identified. In some studies the metabolic activity of the bacterial population as a whole has been examined and individual bacteria have not been enumerated. Any general review of this work is complicated because few papers have considered the same combination of aspects of aquatic bacteriology. Therefore, in this chapter, only a brief outline of previous work and the approaches used will be given. In the chapters describing the experimental work the content of relevant papers is discussed in more detail. References to marine bacteriology are only included where they are pertinent to the bacteriology of freshwater habitats.

In the earlier work on lake bacteria, the emphasis was on bacteria which could be cultured on agar media. The effect of physico-chemical factors on these bacteria in lake water was examined by studying the vertical, horizontal and seasonal variation in their numbers (Fred, Wilson and Davenport, 1924; Graham and Young, 1934). Some more recent papers have also concentrated on the effect of physico-chemical factors on heterotrophic bacteria (Potter and Baker, 1961; Rodina and Kuz'mitskaya, 1963; Guthrie, 1968; Rodina,

1968, 1969; Woodbridge and Garrett, 1969).

The possible importance of biological factors, such as changes in plankton populations, as well as physico-chemical factors in the control of numbers of bacteria was noted by Stark and McCoy (1938), Taylor (1940, 1949), Potter and Baker (1956), Collins (1957, 1960, 1963, 1970), Collins and Willoughby (1962) and Potter (1964), but Henrici (1938), Odum (1957), Silvey and Roach (1964), Chen (1968), Gerletti and Melchiorri-Santolini (1968), Goldman et al. (1968), Overbeck (1968), Sieburth (1968), Štěpánek (1968), Fondén (1969a), Hagedorn (1969), Schegg and Ruschke (1970), Sorokin (1970a) and Jones (1971) combined observations on seasonal or vertical fluctuations in plankton populations with estimates of viable bacteria. The relationship between algae and bacteria in culture experiments has been examined (Waksman, Stokes and Butler, 1937; Steemann Nielsen, 1955; Golterman, 1960, 1964, 1968), and Waksman et al. (1933), Rigomier (1967), Seki (1967) and Simidu, Ashino and Kaneko (1971) have studied the microflora of marine plankton.

The vertical distribution of viable bacteria in mud and the relationship of fluctuations in the bacterial populations of mud to factors, such as temperature, the bacterial population of the water above, and chemical parameters of the water, have been examined (Reuszer, 1933; Williams and McCoy, 1935; Henrici and McCoy, 1938; Carpenter, 1939; Weeks, 1944; Cooper, Murray and Kleerekoper, 1953; Potter and Baker, 1956, 1961; Odum, 1957; Hayes and Anthony, 1959; Anthony and Hayes, 1964; Potter, 1964; Jones, 1971; Nedwell and Floodgate, 1971).

In some studies bacteria have been isolated and

characterized, and attempts have been made to correlate different kinds with changes in environmental factors. A small number of workers have identified freshwater bacteria to genera (Gray, 1951; Rodina and Kuz'mitskaya, 1964; Collins, 1970; Bell, Hoskins and Hodgkiss, 1971; Jones, 1971). Recently the scheme of Shewan, Hobbs and Hodgkiss (1960) for differentiating genera of Gram-negative bacteria has proved useful for identifying isolates from the marine environment (Altschuler and Riley, 1967; Sieburth, 1971). An amended form of the scheme has been published (Hendrie, Hodgkiss and Shewan, 1964).

Although freshwater bacteria have not often been identified to genera, the pigmentation and morphology of isolates have been recorded more frequently. These observations have shown that a predominance of Gram-negative rods and an abundance of pigmented bacteria are typical of the water microflora (Taylor, 1942 and others). In mud a larger proportion of Gram-positive bacteria and fewer chromogens have often been found (Potter, 1964).

Many workers have grouped isolates according to their biochemical abilities. Isolates have been cultured on various selective media (Snow and Fred, 1926; Graham and Young, 1934; Williams and McCoy, 1935; Henrici and McCoy, 1938; Taylor, 1942; Potter and Baker, 1956; Rodina, 1968, 1969; Jones, 1971; Strzelczyk, Antczak and Kuchińska, 1971). The ability of isolates to grow in a range of media of increasing nutritional complexity has also been tested (Potter, 1964; Fondén, 1969b; Strzelczyk, Antczak and Kuchińska, 1971). Fondén (1969a) estimated the lake bacteria on two media, one of which was richer in nutrients than the other.

In this way he distinguished two lake populations with differing nutritional requirements. Strzelczyk and Mielczarek (1971) studied the ability of isolates to oxidize a range of organic compounds.

Studies on the nutritional requirements of bacteria are useful as they indicate the potential activity of bacteria in the lake ecosystem. However, they take into account only those bacteria which are cultured on the original agar plates.

Direct microscopic counts of bacteria have shown how little of the bacterial population is estimated by an indirect method of enumeration. Snow and Fred (1926) concentrated bacteria by using aluminium hydroxide as a flocculating agent. They found that on average their plate counts included only about 11% of the bacterial population as estimated by the direct method. The most commonly used method for concentrating bacteria is by membrane filtration and the disparity between direct counts obtained in this way and plate counts is sometimes very great. As little as 0.005% of the lake water population may be represented by a plate count (Rodina and Kuz'mitskaya, 1963). Jannasch (1965) considered that, depending on the nutrient status of the water, plate counts might represent only 1-10% of the population, and in other studies the numbers of heterotrophic bacteria in water and also in lake sediment have been low compared to the numbers obtained by the direct count method (Hayes and Anthony, 1959; Drabkova, 1965; Sorokin, 1970a and others).

Such results raise the question of whether the difference in counts reflects the presence of a population of

bacteria not considered in plate counts, or whether the difference is mainly due to technical factors such as clumping of bacteria in the sample and the difficulty of distinguishing bacteria from small particles.

Jannasch (1965) considered that technical factors were involved, but that chemosynthetic, photosynthetic and fastidious heterotrophic bacteria, such as Caulobacter, which could not grow on the medium provided, would contribute to the difference between direct counts and plate counts. Goldman et al. (1968) also emphasized that the direct count included a population not estimated by the plate method. In their study of the primary productivity and ecological factors influencing this in Lake Maggiore, these authors found that the bacterial populations estimated by the plate method and the direct microscopic method were correlated with different ecological factors.

In some studies bacteria have been enumerated by the direct microscopic method only. Numbers of bacteria or the biomass of bacteria have been related to fluctuations in plankton populations (Nauwerck, 1963; Overbeck and Babenzien, 1964; Potaenko, 1968; Schegg, 1968; Potaenko and Mikheeva, 1969). More frequently numbers of bacteria have been estimated by both the direct microscopic and plate methods. Rodina and Kuz'mitskaya (1963) and Rodina (1968, 1969) examined the relationship between such bacterial counts and physico-chemical factors of lakes. Fluctuations in plankton populations or other biological factors and bacterial populations, estimated directly and indirectly, were considered by Odum (1957), Gerletti and Melchiorri-Santolini (1968), Goldman et al. (1968), Overbeck (1968), Štěpánek

(1968), Schegg and Ruschke (1970), Sorokin (1970a) and Tezuka (1970).

The need for an accurate measure of bacterial biomass has led to the development of a method whereby the amount of ATP is estimated (Holm-Hansen and Booth, 1966). Of particular value, however, when the role of bacteria in lakes is being studied, are the methods in which the metabolic activity of the bacterial population as a whole is determined. When combined with direct and indirect counts of bacteria, these methods are the most promising for clarifying the role of bacteria in lake water.

Drabkova (1965) determined the generation time of bacterial populations by studying their rate of multiplication in bottles with and without the natural zooplankton populations. She obtained a figure by which different water samples could be compared and related this to direct and indirect counts of bacteria. Tezuka (1970) compared the respiration rates of the total plankton population and the bacterial population by measuring oxygen uptake after incubation in situ or at a similar temperature for 15 to 24 h. The bacteria were separated from the rest of the plankton by filtration through an 8 μm pore-size filter.

Radioactive isotopes have been applied in several ways to the study of bacterial activity in aquatic environments. The ^{14}C-carbonate method of Steemann Nielsen (1952), which has become a standard method of estimating the productivity of phytoplankton, was developed by Parsons and Strickland (1962) to study heterotrophic uptake of glucose and acetate in sea water. In this way an estimate of the minimum value for the true heterotrophic uptake of carbon - the 'relative

heterotrophic potential' of the sample - can be obtained. Sorokin (1969, 1970b), using the method of Parsons and Strickland, obtained a relative figure for the metabolic activity of bacterial populations in mud and sea water. He combined the technique with plate counts of heterotrophic bacteria.

Hobbie and Wright (1965) and Wright and Hobbie (1965, 1966) revised the method of Parsons and Strickland and, using glucose and acetate as substrates, studied the uptake kinetics of freshwater bacteria at different concentrations of substrate. By their method it is possible to measure the turnover rate of certain compounds in situ. The potential heterotrophic ability of the population is also determined. A similar approach was used by Vaccaro and Jannasch (1966) to study heterotrophic activity in sea water. Allen (1969) studied the seasonal variation in the uptake of radioactive glucose and acetate by planktonic algae and bacteria in a pond. The metabolic activity of epiphytic bacteria and algae on macrophytes has also been examined using ^{14}C techniques (Allen, 1971).

Radio-isotopes have also been used to study the role of bacteria in the turnover of minerals such as phosphorus. Such studies include those of Hayes and Phillips (1958) who examined the rate at which ^{32}P added to water equilibrated between mud, plants and bacteria, and Johannes (1965), who investigated the uptake and excretion of phosphorus by zooplankton and the mineralization of soluble inorganic phosphate from detritus.

Studies on the metabolic activity of the bacterial population as a whole do not distinguish between the inactive

and active bacteria of the population. The stain, acridine orange, with fluorescence microscopy has been used to distinguish between live and dead microorganisms (Strugger, 1948) but many workers have had difficulty interpreting material stained in this way (Wood, 1963). The metabolic activity of individual bacteria can be examined using autoradiographic methods (Brock and Brock, 1966, 1968). These methods were combined with the technique of Wright and Hobbie (1966) in a study of uptake of acetate by bacteria and algae attached to sand grains on a littoral beach (Munro and Brock, 1968). Paerl and Goldman (1972) included autoradiograms of lake bacteria in their investigation of mass movement of water which had originated from a stream flowing into the lake, and the spread of radioactive bacteria in water has been detected by autoradiography (Berlin and Rylander, 1963). Quantitative results can be obtained from autoradiograms by counting the proportion of labelled and unlabelled cells (Brock and Brock, 1968).

The various approaches of these studies have shown that aquatic bacteria are influenced by many factors but the basic question of what proportion of aquatic bacteria are mineralizing organic matter still remains to be answered.

1.4 OBJECTIVES OF THE STUDY

No ecological study can consider the effects of all variables on an organism or group of organisms. In the present study the heterotrophic bacterial population of one lake was considered. The basic aims were to determine what factors were influencing the bacterial populations in a number of habitats within the lake, and how the activity of these

bacterial populations was related to the trophic status of the lake.

In much of the work bacteria were counted by the dilution plate method. The bacteria estimated by this method represent a part of the bacterial population which is viable and potentially active. The data obtained by studying their response to changes in certain factors of the environment give an indication of the extent to which these bacteria are responsible for the mineralization of organic matter in different habitats within a lake.

The seasonal fluctuations in numbers and kinds of heterotrophic bacteria in the open water of the lake were considered in relation to:-

a) amount of rainfall, numbers and kinds of bacteria in certain inlets and nutrient concentrations in the open water and inlets;

b) numbers and kinds of bacteria on Elodea canadensis and in lake water over Elodea;

c) changes in plankton populations;

d) numbers and kinds of bacteria in the mud.

CHAPTER 2

MATERIALS AND METHODS

2.1 DESCRIPTION OF LAKE

The lake studied was Lake Grasmere (Fig. 2.1) which has been described by Stout (1969, 1972). Details of isobaths and the country surrounding Lake Grasmere are taken from the map of Irwin (1969).

> "Lake Grasmere is situated in the glaciated mountain region of Canterbury...at an altitude of approximately 600 m above sea level.
>
> The lake has an area of approximately 63 hectares and a maximum depth of 12 m. It lies in the path of the prevailing northwesterly wind and the water is frequently mixed by strong wind action. Temperature stratification is not established in the summer, except occasionally for short periods of time during calm weather. Ice may form over all or part of the lake during winter. The water is always well oxygenated.
>
> Most of the land surrounding the lake is tussock country, and much of it is now used for agriculture, including the growing of legume crops and grazing of stock" (Stout, 1972).

Along the northeast side of the lake are patches of bush and forest, in which the dominant species is Nothofagus solandri var. cliffortioides (Hook. f.) Poole.

Within the lake are extensive weed beds of Elodea canadensis Michx.. A series of diving transects, carried out in April, 1970, showed that Elodea was growing down to depths of 9 m but usually only to about 6-7 m around most of the lake (Fig. 2.1). Among the Elodea were patches

of scattered growth of Ranunculus, Potamogeton, Myriophyllum, Isoetes and Nitella.

The annual rainfall varies from year to year. During the period of study, the rainfall was as follows:-

1969 - 93.2 cm

1970 -118.0 cm

1971 - 92.4 cm.

Water drains through alluvial fans and the main inflow is through one small stream and a number of springs. Three of these inlets, which were studied, are shown in Fig. 2.1. Both C, the small stream, and A, a spring, flow into the south side of the lake. C consists of a cutting about 1 m wide and 0.5 m deep. It passes through grassland frequently grazed by stock, and is often silty. Spring A flows out of swampy ground about 50 m from the lake and forms a pool approximately 0.5 m deep, which is often a source of water for cattle. Inlet H is representative of the water draining from the catchment area to the northwest of the lake. This inlet is a spring, which flows into the lake in the harbour, beneath 2 m of water. The main source of water for A and C is probably a stream (not shown on the map) at the head of the fan on the south side of the lake. This stream flows through a 30 m wide shingle bed but the stream itself is only 1-2 m wide, except after heavy rain. The water from this stream does not flow directly into the lake but percolates through the fan.

The nutrient status of Lake Grasmere is considered to be oligotrophic to mildly eutrophic and large quantities of organic matter, phosphates and nitrates are not brought into the lake. Before 1968, no limnological studies had been

made of Lake Grasmere and it is not known how long Elodea canadensis has been growing in the lake. Elodea was first introduced to Canterbury by the Canterbury Acclimatisation Society, who planted it in a pond near Christchurch in 1868. In 1968, Dr V.M. Stout began sampling the lake regularly as part of the I.B.P. freshwater programme. From the open water station at 12 m (Fig. 2.1), observations were made at different depths, including temperature, light penetration, oxygen content, nutrients and plankton. Samples from inlets were also studied.

The initial year's sampling showed that the bacterial population of the open water, as estimated by the pour plate method, fluctuated around 300 bacteria per ml.

2.2 SAMPLING STATIONS AND TECHNIQUES

Water

Open water, water over the Elodea canadensis beds and over an inflowing spring were regularly sampled from the lake.

Open water was sampled from water 7 m or more in depth away from the shore and weed beds. There was no fixed sampling station, but samples were taken from within either of the areas 'OW' in Fig. 2.1. The sampling depth was 3 m except in the first two experiments, when samples were taken at 1 m.

Water, from just above the weed bed, was sampled from within the areas 'W' and 'W 6' shown in Fig. 2.1. At the regular sampling station (W) the total water depth was 2 to 3 m and water was sampled at about 0.75 m. In one

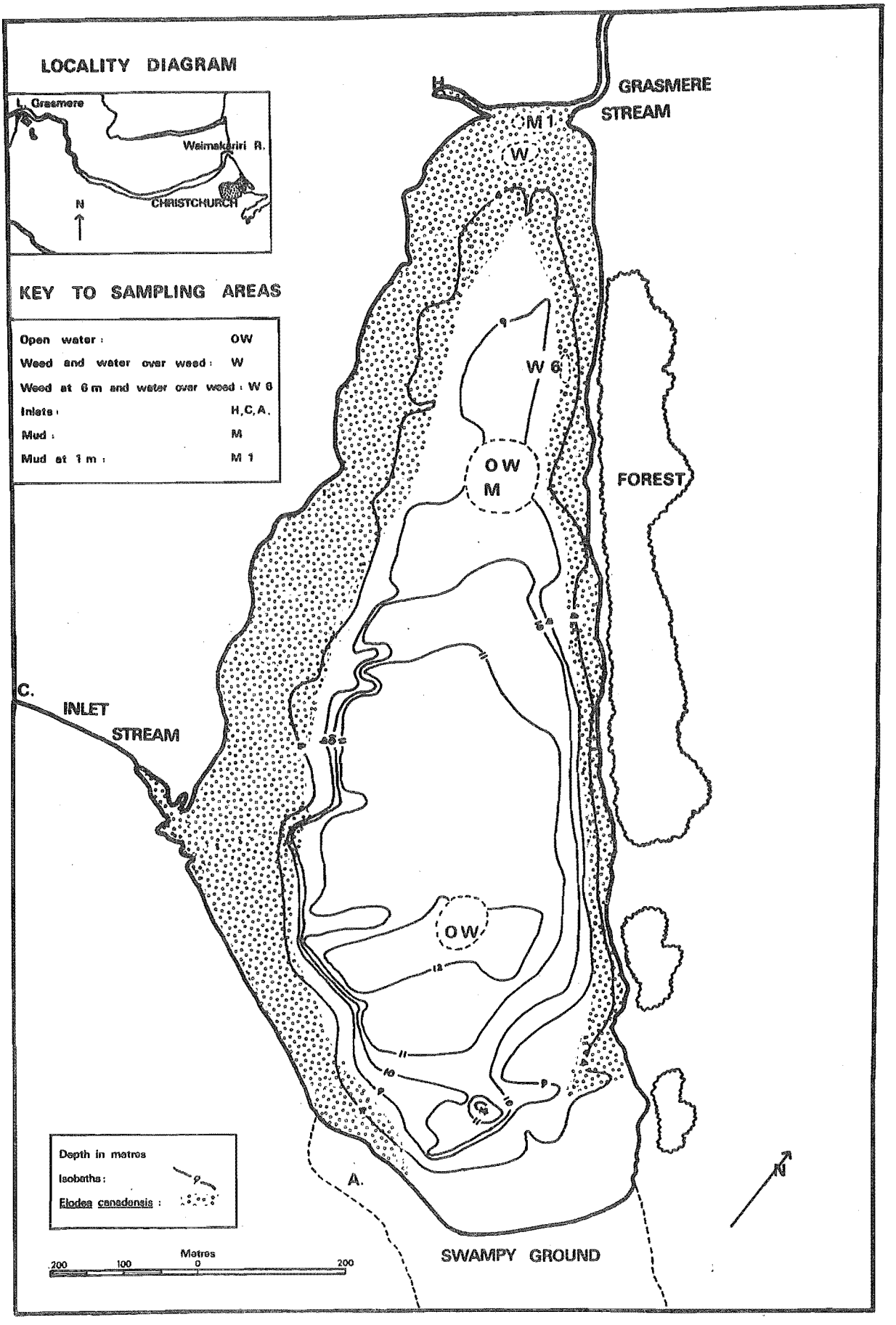


FIG. 2.1 Map of Lake Grasmere showing sampling areas

experiment water over weed growing at 6 m was sampled.

Water over the inflowing spring H in the northwest corner of the lake (Fig. 2.1) was sampled from within 0.3 to 0.4 m of the spring inflow.

Inlet A (Fig. 2.1) and stream E, the presumed source of A and C (not shown on the map), were sampled only once and inlet C (Fig. 2.1) was sampled four times. C was sampled about 200 m from the lake. The sample of spring A was taken at its source and E was sampled about 30 m above the point where it went underground.

Most of the water for bacteriological analysis was sampled aseptically using a modified J-Z sampler (Zobell, 1946). The sampler, which was made from P.V.C., is illustrated in Fig.2.2. The two main modifications were the introduction of a double lever system to hold the capillary tubing in place, and a T joint to allow an extra outlet so that the bottle could be evacuated after autoclaving. Before evacuation, all joints were sealed with Prestik Builders' sealing strip (Bostik, N.Z., Ltd.). Samples contained between 80 and 220 ml of water.

In two preliminary experiments and in some of the later experiments, a one litre perspex Ruttner sampler was used. The samples collected for bacteriological analysis were also used to study algae. Zooplankton samples were obtained with the Ruttner sampler.

Inlets, C and A, and stream E were sampled by opening a sterile one litre bottle beneath the surface of the water.

A single sample was collected from each site on each occasion.

FIG. 2.2 Modified J-Z sampler

Elodea canadensis

Weed growing in 2 to 3 m of water and, in one experiment, in 6 m of water (Fig. 2.1, 'W' and 'W 6') was collected with a four prong weed grab and placed in a plastic bag. The samples included all parts of the plants.

Mud

Mud was usually sampled beneath open water at a depth of 7 to 9 m (Fig. 2.1 'M'). In two experiments mud was sampled among the weed at a depth of 1 m (Fig. 2.1, 'M 1'). An Ekman-Birge bottom grab was used which had an aperture 15x15 cm. This sampled the mud to a depth of about 18 cm, but only mud from about the top 5 cm of this sample was taken for analysis.

All samples were placed in an iced container until they were treated either at a field station about 5 km from the lake or in the laboratory.

2.3 ENUMERATION OF VIABLE BACTERIA

Method of enumerating bacteria

The pour plate method was used to enumerate bacteria. In retrospect, the streak plate method might have been more suitable as heat injury to the bacteria would have been avoided. However, van Soestbergen and Ching (1969) found that bacterial counts obtained with the streak plate method were less precise than those obtained with the pour plate method. They attributed this to the variation in the numbers of bacteria remaining on the spreader.

In 1969, when the choice of method was made, the main

disadvantage of the streak plate method appeared to be the requirement of a small inoculum volume, usually of 0.1 ml. As the bacterial population in the water was sometimes low, inoculum volumes of as much as 1 ml would sometimes have been necessary to ensure sufficient colonies grew on each plate. Cowell and Morisetti (1969) noted that it was customary to count plates with between 30 and 300 colonies. As there were some conflicting reports on which method gave the highest counts, the pour plate method was chosen. Buck and Cleverdon (1960) reported higher bacterial counts using surface inoculation but Carlucci and Pramer (1957) found that 30 to 40% more colonies developed on pour plates. Jannasch and Jones (1959) noted that there was little difference between pour plate counts and counts of colonies growing on membrane filters placed on agar.

More recently, Jones (1970) showed that the streak plate method was more suitable for estimating freshwater bacteria and Clark (1971) has satisfactorily surface-inoculated plates, which had previously been dried, using inoculum volumes up to 1 ml.

Analysis of pour plate counts

Theoretically, counts of replicate pour plates will form a Poisson distribution provided the sample is homogeneous and each plate is prepared in the same way (Fisher, Thornton and Mackenzie, 1922). The chi-square test can be used to test the goodness of fit of plate counts with Poisson expectations and has become a test of the reliability of such data (Eisenhart and Wilson, 1943). These authors noted that large variances resulting in large chi-squares were often found when

complex bacterial populations such as those present in soil were studied. They attributed these discrepancies to faults in technique and to interactions between microorganisms and considered that data giving unusually large or small chi-square values were suspect. More recently in a comparison of colony counts on membrane filters of bacteria from fresh and frozen estuarine sediment, Anthony (1970) discussed the need to discard certain counts on the basis of the chi-square test. He concluded that the failure to achieve randomly distributed counts was due to factors other than technique and that all the data should be analyzed after a suitable transformation.

If 't' tests are to be applied to data or correlation coefficients calculated, one basic requirement is that data should form a normal distribution, ie. that the mean should be independent of the variance (Snedecor and Cochran, 1967). In the case of a Poisson distribution the mean is equal to the variance but it is made independent by a square root transformation of the raw data (Snedecor and Cochran, 1967). Where data do not form a Poisson distribution there is the problem of determining the appropriate transformation. The Power Law of Taylor (1961) has been used to determine suitable transformations of colony counts of bacteria from lake mud and water (Anthony, 1970; Jones, 1971). To obtain the transformation, log variance on log mean are plotted and the regression line calculated. The required transformation of x is $x^{(1-b/2)}$, where b is the gradient of the line of regression. In the present study the chi-square test was applied to some of the field data and this showed that although some counts formed Poisson distributions, a number did not.

This necessitated the calculation of a transformation suitable for the data which were to be analyzed statistically. Eighty-two sets of plate counts from water samples only were used to calculate b. The correlation coefficient for log variance and log mean was r = 0.752 and the suitable transformation was $x^{0.24}$. These results were similar to those obtained by Anthony (1970), viz. frozen sediment r = 0.756, $x^{0.21}$; fresh sediment r = 0.717, $x^{0.27}$, and Jones (1971), who found the appropriate transformation for estimates of viable aquatic bacteria was $x^{0.2}$.

The best methods for enumerating bacteria were determined from a number of plate counts which were analyzed with 't' tests, after square root transformations of the data. When chi-square tests were applied later and it was found some of the data did not conform to a Poisson distribution, the data were reanalyzed using the transformation $x^{0.24}$. The same conclusions were reached with this second analysis.

Correlation coefficients were calculated between mean plate counts of bacteria in the lake water and chemical parameters or rainfall. The individual plate counts were transformed ($x^{0.24}$) and 'transformed means' obtained. As counts from 10^{o}, 10^{-1} and 10^{-2} dilutions were used, before the correlation coefficients were calculated all data were converted to 10^{o} dilution by multiplying the 'transformed means' by $10^{0.24}$ or $10^{0.48}$ where necessary.

The chi-square test was applied to plate counts of pure cultures of bacteria to check that they formed a Poisson distribution. Following a square root transformation of the plate counts, the means were compared using 't' tests.

In all Tables which include plate count data, the means

and standard deviations of the counts are given.

The pour plate method

Samples were diluted by 10- or 100-fold steps with distilled water. Straka and Stokes (1957) noted the destructive effect of distilled water as a diluent, but in the present study the small dilution factor of the water samples and the presence of mud particles or leaf material in the dilutions would reduce this effect because of the additional nutrients and surfaces for bacterial attachment present.

Agar at 48°C was poured on to 1 ml of inoculum and thoroughly mixed with it. The main disadvantage of this method is that the temperature of the molten agar may be high enough to kill some of the bacteria before the agar sets and cools down. Gunkel, Jones and Zobell (1961) showed that numbers were least reduced when a 10 ml volume of agar was used. Glass Petri dishes were preferable to plastic ones as the agar cooled down more quickly in these. These conditions were followed in this study.

In the first experiment three replicate plates were set up, but for the remainder of the experiments five replicates were used.

Treatment of individual samples

All samples and subsequent dilutions were shaken for 20 s by hand, unless stated otherwise.

a) Water

Samples were diluted by 10-fold steps with 90 ml water blanks. Undiluted lake water and one or two dilutions were

plated out. Plate counts were expressed as the number of bacteria per ml of water.

b) Mud

Five gram samples in 45 ml of water (dilution 1) were shaken mechanically for 5 min. The initial dilution was diluted 100-fold by transferring 1 ml of dilution 1 to a 99 ml blank (dilution 2, plated). Ten ml of dilution 2 was added to a 90 ml blank and this third dilution was also plated out.

Plate counts were expressed as the number of bacteria per g dry wt of mud. The dry weight was determined by drying a sample to constant weight at 80°C.

c) Elodea canadensis

Five gram samples were macerated in 100 ml of water (dilution 1) with a homogenizer (Measuring and Scientific Equipment Ltd., England; vortex type with speed up to 14 000 rev/min) for 2 min at half full speed and 2 min at three-quarter full speed. This blendor has the motor placed over the maceration flask. Chan and McManus (1967) found that homogenization of algal samples with a Waring Blendor, in which the motor is beneath the flask, resulted in the death of some bacteria due to a combination of heat and mechanical injury. They advocated the use of a blendor in which the maceration flask was not situated over the motor, and was thus less likely to heat up during maceration. After macerating the sample, further dilutions were prepared as follows: 5 ml of dilution 1 into a 45 ml blank (dilution 2), 1 ml of dilution 2 into a 99 ml blank (dilution 3, plated), 10 ml of dilution 3 into a 90 ml blank (dilution 4, plated),

and 10 ml of dilution 4 into a 90 ml blank (dilution 5, plated). Plate counts were expressed as the number of bacteria per g fresh wt of Elodea.

In one experiment the numbers of bacteria were estimated per mm^2 of leaf surface. Thirteen leaves were sampled from a selected region of a stem. Ten of these leaves were macerated in a 10 ml water blank (dilution 1) for 4 min as above and diluted further by transferring 0.1 ml of dilution 1 to a 9.9 ml blank (dilution 2); 1 ml of dilution 2 to a 9 ml blank (dilution 3, plated); 1 ml of dilution 3 to a 9 ml blank (dilution 4, plated); and 1 ml of dilution 4 to a 9 ml blank (dilution 5, plated).

Plate counts were expressed as the number of bacteria per mm^2 of leaf surface. The surface area was determined from the remaining three leaves of the sample by drawing round the leaves on mm square graph paper and, from the outlines, calculating the average surface area of a leaf.

d) Plankton

A sample consisting predominantly of algae was obtained by centrifuging 10 ml of lake water for 5 min at 650 g. The supernatant was decanted off and made up to 10 ml with sterile water. It was diluted in the same way as the lake water samples and plated out. The remaining pellet in 0.1 to 0.2 ml water was made up to the original volume of water and the plankto resuspended. The bacteria in the suspension were plated out.

An estimate of the bacteria associated with the zooplankton was obtained by plating out material concentrated in a net. A known volume of water was passed through a No. 15 net (mesh-size, 94 μm). The plankton, which consisted of

all the zooplankton and some of the algae in the original sample, was collected in about 50 ml of water. This was made up to 100 ml and macerated for 4 min (dilution 1) before diluting 5 ml with 45 ml of water (dilution 2), and 1 ml of dilution 2 with 9 ml of water. All three dilutions were plated out.

Plate counts were expressed as the number of bacteria associated with plankton per ml of undiluted lake water. The counts were corrected to allow for any bacteria suspended in the water containing the concentrated plankton as described on pp.222 and 223.

Suitability of medium

One of the basic requirements of a nutrient medium, which is to be used to enumerate bacteria over a period of time, is that the composition of the medium should vary as little as possible from one batch to another. This excluded the use of lake water in the medium as the nutrients in the lake water would vary over the sampling period of two and a half years.

Other conditions which the medium had to satisfy were that

(i) it must give a high count combined with the greatest diversity of kinds;

(ii) the medium must be one from which the bacteria could be successfully isolated;

(iii) the medium must fulfil these requirements for bacteria from water samples and also, to as great an extent as possible, for bacteria from other habitats within the lake, e.g. mud, Elodea. This last condition was made so that the

bacterial populations examined from habitats other than lake water would be directly comparable with the water population.

Three media were tested:

(i) casein, peptone, starch medium (CPS) described by Collins and Willoughby (1962) except that sodium caseinate (0.5 g/1) was used instead of casein (CA). The CPS medium was compared with seven others by Taylor (1940) and gave the highest counts. More recently, Jones (1970) compared the CPS medium with Plate Count Agar and tryptone-glucose-extract, both recommended by the American Public Health Association (1965) for testing water quality. He found that, with incubation for up to 23 days, CPS was the most suitable of the three media for enumerating freshwater bacteria;

(ii) yeast extract-peptone agar diluted 25 times (YPA/25). Fondén (1968) compared a range of dilutions of yeast extract-peptone agar with three media containing sodium caseinate and found that the yeast extract-peptone agar diluted 25 times, which contained 0.2 g peptone, 0.12 g yeast extract, and 15 g agar per litre distilled water, gave the highest counts of bacteria for samples from a slightly enriched lake;

(iii) nutrient agar (NA) - Oxoid nutrient broth (Code No. CM1) and 15 g agar per litre distilled water.

The agar for all solid media was Davis bacteriological agar (Davis Gelatine (N.Z.) Ltd. Christchurch).

Open water, water over weed and mud were sampled in April and June, 1969. The April samples were plated on all three media but in June only NA and YPA/25 were used. The numbers of bacteria per ml of lake water and per g dry wt of mud were

determined. The significance of the differences between plate counts from each sample using different media was tested as described on p.21 (Table 2.1). From plates of each medium, 40 bacteria were isolated, characterized and grouped as described on pp. 32-45. This led to the distinction of 12 groups. The number of different groups of bacteria and the number of these groups which contained more than 5% of those characterized were determined (Table 2.1). The latter distinction indicated how many of the groups of bacteria made up a significant part of the population.

The counts of water, sampled in April, using CA were not significantly different from those using either of the other two media. Only a single plate was counted with the mud sample using CA. This count was higher than those obtained with the other media but could not be compared statistically. An examination of the range of different kinds of bacteria found on CA showed that for the two water samples the number of groups of bacteria making up a significant part of the population was much lower than on NA or YPA/25. This trend was reversed with the mud sample. From the results obtained in April it was decided that, as a narrower range of bacteria was isolated from water samples plated on CA and the counts were not significantly higher than those obtained with the other two media, this medium would not be tested further. The results suggested that CA might have been the best medium for estimating the numbers and kinds of mud bacteria but it was not so suitable for enumerating bacteria in the water of Lake Grasmere.

Differences between mean plate counts were significant for only 3 of the 11 pairs of means tested. In each of these

TABLE 2.1 Effect of different media on estimation of numbers and kinds of bacteria

Date sampled	Sample	Medium	No. bacteria (per ml)	No. different groups of bacteria	No. groups comprising more than 5%
		NA	380 ± 100	7	6
April	open water	YPA/25	300 ± 20	6	5
		CA	245 ± 30	8	2
June	open water	NA	440 ± 120[1]	9	6
		YPA/25	880 ± 390[1]	8	5
April	water over weed, plated within 1.5 h	NA	1090 ± 70	8	6
		YPA/25	1130 ± 160	7	6
April	water over weed, plated within 7 h	NA	730 ± 55[1]	7	5
		YPA/25	1020 ± 125[1]	5	4
		CA	840 ± 70	6	2
June	water over weed	NA	580 ± 170	7	5
		YPA/25	825 ± 570	7	5
			(per g dry wt x 10^{-6})		
		NA	2.19 ± 1.02	5	4
April	mud	YPA/25	3.14 ± 1.3	7	4
		CA	5.85 x	6	6
June	mud	NA	2.04 ± 0.28[2]	9	4
		YPA/25	7.0 ± 1.07[2]	4	3

x Only one plate was countable.

[1] Difference significant at 5% level; [2] Difference significant at 1% level; All other differences were not significant.

three cases, YPA/25 gave significantly higher counts than NA but for the four other samples when NA and YPA/25 were compared the differences were not significant (Table 2.1). From the results of the April experiment it appeared that while YPA/25 tended to give higher counts, a greater diversity of bacteria grew on NA. The June experiment confirmed these observations although there was little difference in the number of groups of bacteria isolated from both media, which made up a significant part of the population.

However, there was one major disadvantage of YPA/25. Although bacteria from the water samples were readily cultured from YPA/25, only 45% of the isolates from the mud sampled in April and 47% of those from the mud sampled in June grew. If counts of bacteria only had been required, YPA/25 would probably have been more suitable, but as it seemed to be unreliable for estimating the kinds of bacteria in mud samples, it did not satisfy the three requirements listed on p.25 as well as NA. Therefore NA was used throughout the remainder of the work.

Temperature of incubation

All pour plates were incubated at 20°C. This was within the temperature range of the lake over the sampling period (2.2 to 21.2°C).

Incubation period

In the first experiment the numbers of colonies per plate of NA were counted after 6, 10, 14, 17 and 21 days (Fig. 2.3). All counts were made with the aid of a Gerber counter (Karl Kolb Scientific-Technical Supplies, Germany).

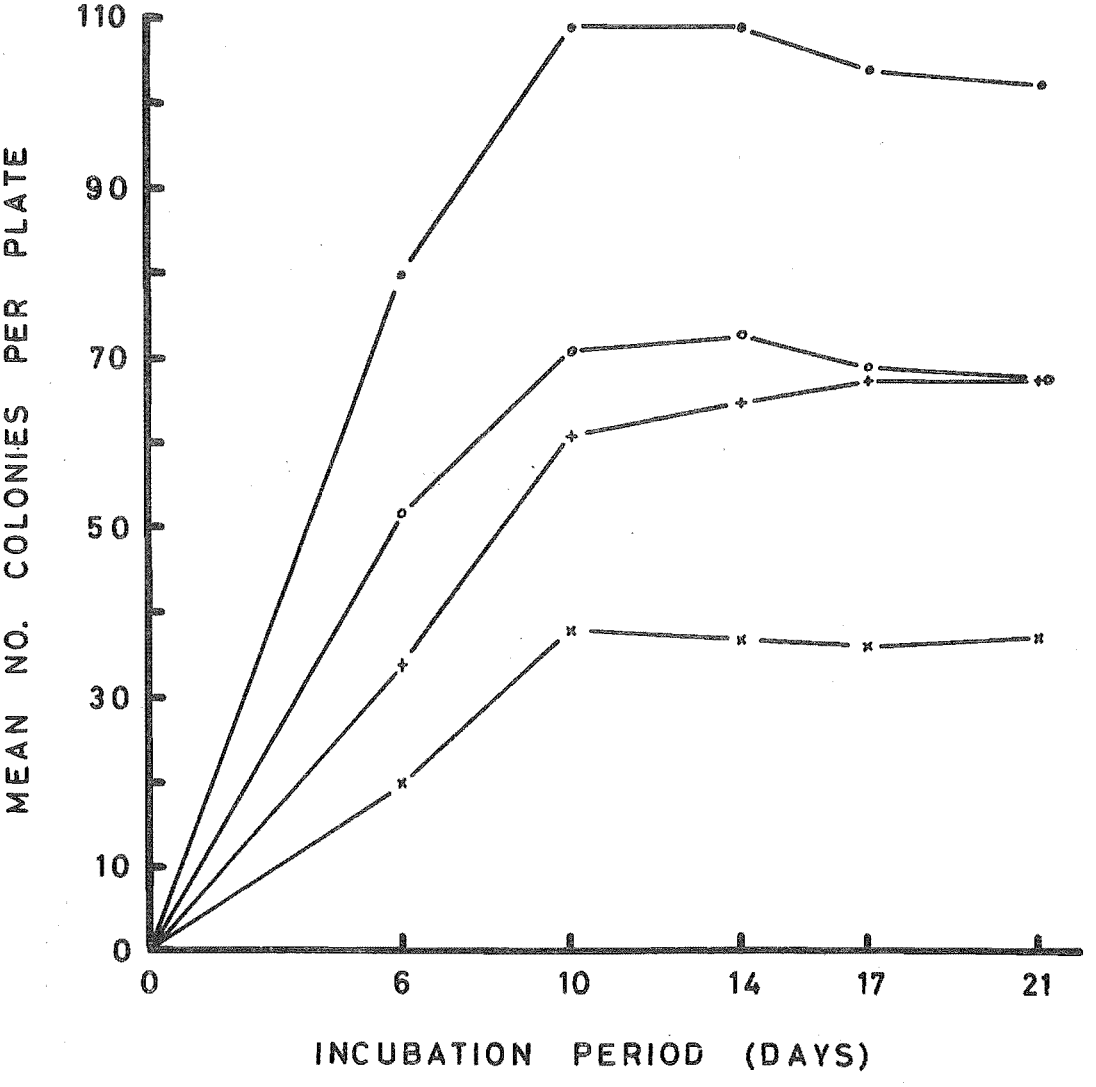


FIG. 2.3 Effect of incubation period on plate counts of bacteria on NA. Open water —x—; mud —+—; water over weed plated within 7 h —o—; water over weed plated within 1.5 h —•—.

For the water samples there was no increase in the numbers of colonies per plate after 14 days incubation. The numbers of colonies from the mud increased slightly between 14 and 17 days incubation. In later experiments plates were incubated for 14 days. Similar counts on YPA/25 and CA showed that up to 21 days incubation was needed to ensure a maximum count.

Time lapse between sampling and plating

Water over weed sampled on 15 April, 1969, was plated out within 1.5 and 7 h of sampling on two media. The changes in plate counts and kinds of bacteria were examined (Table 2.2). An analysis of the data showed that while a time lapse of up to 7 h before plating produced changes in the total numbers, these were significant in only one of the two cases. The time lapse of up to 7 h before plating did not appear to select for any group of bacteria, but fewer groups were isolated from both media when the water was not plated shortly after sampling. It was decided that as a time lapse of up to 7 h could produce a significant change in the bacterial population, plating within 2 h would ensure any change was minimal.

Therefore all water sampled after the first experiment was plated within 2 h of sampling. Samples of weed, mud and plankton, in which the bacteria were attached to a substrate, were less likely to be affected by storage. These were plated out within 7 h of sampling on returning to the laboratory.

TABLE 2.2 Effect of time lapse between sampling and plating on the numbers and kinds of bacteria in water samples

Sample	Maximum time between sampling and plating (h)	No. bacteria (per ml)	No. different groups of bacteria	No. groups comprising more than 5%
Water over weed, NA	1.5	1090± 70[1]	8	6
	7	730± 55[1]	7	5
Water over weed, YPA/25	1.5	1130± 160[2]	7	6
	7	1020± 125[2]	5	4

[1] Difference significant at 1% level;

[2] Difference not significant.

Summary of pour plate method used for enumerating viable bacteria after April and June experiments

One ml of inoculum was mixed with 10 ml of nutrient agar in pyrex Petri dishes, within 2 h sampling for the water samples and within 7 h of sampling for the samples of mud, weed and plankton. Five replicates were set up for each dilution. Plates were incubated at 20°C for 14 days.

2.4 CHARACTERIZATION OF BACTERIA

A range of bacteria from 54 samples was characterized so as to compare the kinds of bacteria in samples collected at different times of the year and from different habitats.

With any characterization of bacteria there must be a compromise between the number of bacteria isolated and the number of tests used to characterize them. In this work sufficient tests were required to identify bacteria to recognized groups, either family or genus, so that the results obtained could be compared with previous studies. However, three groups of bacteria, as noted below, were identified in more detail.

All routine tests on the bacteria, except for the examination of flagella using the electron microscope (EM), were carried out within four weeks of isolation from the pour plates used to enumerate the bacteria. This ensured that bacteria were characterized before any possible changes in biochemical behaviour with maintenance as a stock culture occurred. Also there was less chance that some of the cultures might die before all tests had been completed. The number of bacteria isolated from each sample was made as large as could be practically handled in the time available.

Thus, after the two preliminary experiments, 50 bacteria were usually isolated from each sample. In the preliminary experiments each sample was grown on several media and, because of the increased numbers of treatments, 40 bacteria were isolated from each treatment. In all, 2515 bacteria were isolated from field samples and some laboratory experiments and, of these, 2112 were characterized. The majority of the remainder were not viable after the first transfer. A few were actinomycetes or yeasts, which were not included in this study, or mixed bacterial cultures.

Isolating a larger number of bacteria would have increased the chance of including any bacterium which was present in only

small numbers. However, with the limited number of tests used and number of groups of bacteria differentiated, it is quite possible that no further groups would have been detected. Enrichment is one method of detecting low numbers of specific groups of bacteria and two media which were used to test for the presence of cellulose- and methanol-utilizing bacteria are described on p. 52.

For the routine characterization of bacteria up to 12 characters were recorded. The incubation temperature was 20°C.

Bacteria from all discrete colonies in a Petri dish, or from a sector chosen at random, were isolated on to either nutrient agar (Oxoid) or the glucose-yeast extract-chalk agar (GYCA) described by Dye (1962). After incubation for 2 to 4 days the culture was examined to ensure colonies were not mixed.

Stock cultures were maintained on nutrient agar slants. Any cultures which were to be kept for more than 3 or 4 weeks were stored under mineral oil at room temperature.

In the characterization of the cultures, the following techniques were used:-

Pigmentation and colony appearance (including formation of spreading colonies): These characteristics were noted on cultures incubated for 4 days on GYCA.

Motility: 18-24 h cultures in nutrient broth were examined with phase-contrast illumination.

Flagellation: A selection of motile bacteria from 18-24 h nutrient agar slants was negatively-stained with 2% phospho-

tungstic acid (PTA) (Horne, 1965). A suspension of bacteria in filter-sterilized, distilled water was diluted with an equal volume of PTA and left for 7-8 min. The stained cells were then applied to carbon-coated colloidin grids by touching a one-hole grid on to the suspension and lowering this on to the grid. The suspension was immediately dried down on to the grid with a corner of filter paper. The bacteria were examined under a Hitachi HS-7 EM.

Gram reaction and morphology: Heat-fixed smears of bacteria from 18-24 h nutrient broth cultures were Gram-stained as described by Eklund and Lankford (1967).

Oxidase: The method of Kovacs (1956) was used on cultures incubated for 4 days on GYCA.

Catalase: The presence of the enzyme catalase in some of the cultures was tested using any one of the methods described by Harrigan and McCance (1966).

Oxidative-fermentative attack of glucose: The medium of Hugh and Leifson (1953) was used. Two bijou bottles containing 4-5 ml of medium were stab inoculated and one was sealed with vaspar. They were incubated for up to 14 days and the formation of acid recorded three times - after 2 to 4 days, 5 to 8 days and 12 to 14 days. The long incubation period was used because some bacteria could utilize glucose only slowly.

On the basis of these tests, the bacteria were grouped into broad categories and further tests were carried out on bacteria in only some of these categories.

Formation of a diffusible, fluorescent pigment: Bacteria were

streaked on to Medium B of King, Ward and Raney (1954) but using Difco Bacto tryptone instead of proteose peptone. After incubation for 2 to 4 days, cultures were examined under u.v. light.

Production of ammonia from arginine: The ability to produce ammonia from arginine fermentatively was tested using Medium 2A and the method of Thornley (1960).

These two tests were confined to some of the Gram-negative, non-pigmented, motile isolates.

Cellulose degradation: Using a mineral salts medium (No.2) with filter paper as the source of cellulose (Harrigan and McCance, 1966) a number of the isolates pigmented yellow or orange were tested for the ability to degrade cellulose.

Using the results of all the above tests, isolates were divided initially into 12 groups. The schemes of Shewan, Hobbs and Hodgkiss (1960) and Shewan (1963) were used to differentiate the genera of Gram-negative bacteria. The scheme of Shewan (1963) is essentially the same as the 1960 scheme, but is applicable especially to bacteria from a marine environment. However, certain anomalies were apparent in the schemes and some different groups were recognized in this study. When the study had been completed, the paper of Hendrie, Hodgkiss and Shewan (1964), in which these schemes were amended, was consulted. This modified scheme is more complex and removes several of the anomalies by including some non-motile bacteria in the Enterobacteriaceae and recognizing certain motile bacteria with peritrichous flagella as Alcaligenes or Achromobacter. It would appear to be far

more satisfactory for identifying Gram-negative rods from an aquatic environment than the original scheme. The modifications to the earlier scheme relating to the Enterobacteriaceae and Alcaligenes/Achromobacter, which were made in this study, were essentially the same as those of Hendrie, Hodgkiss and Shewan (1964).

The Gram-positive bacteria, which made up a minor part of the population, were divided into three groups based mainly on the morphology of the cells.

An outline of the scheme used to identify the bacteria is given in Table 2.3. Details of the characteristics of the individual groups recognized are as follows:

a) pseudomonads

All bacteria which fell into Pseudomonas Groups I,II,III and IV according to the scheme of Shewan, Hobbs and Hodgkiss (1960) were grouped together in this study. Because such a diversity of bacteria was included in this group, the bacteria were designated pseudomonads rather than Pseudomonas. When 24 isolates, which on the basis of biochemical tests, Gram reaction and motility fell into the group pseudomonads, were examined under the electron microscope, it was found that eight had peritrichous flagella.

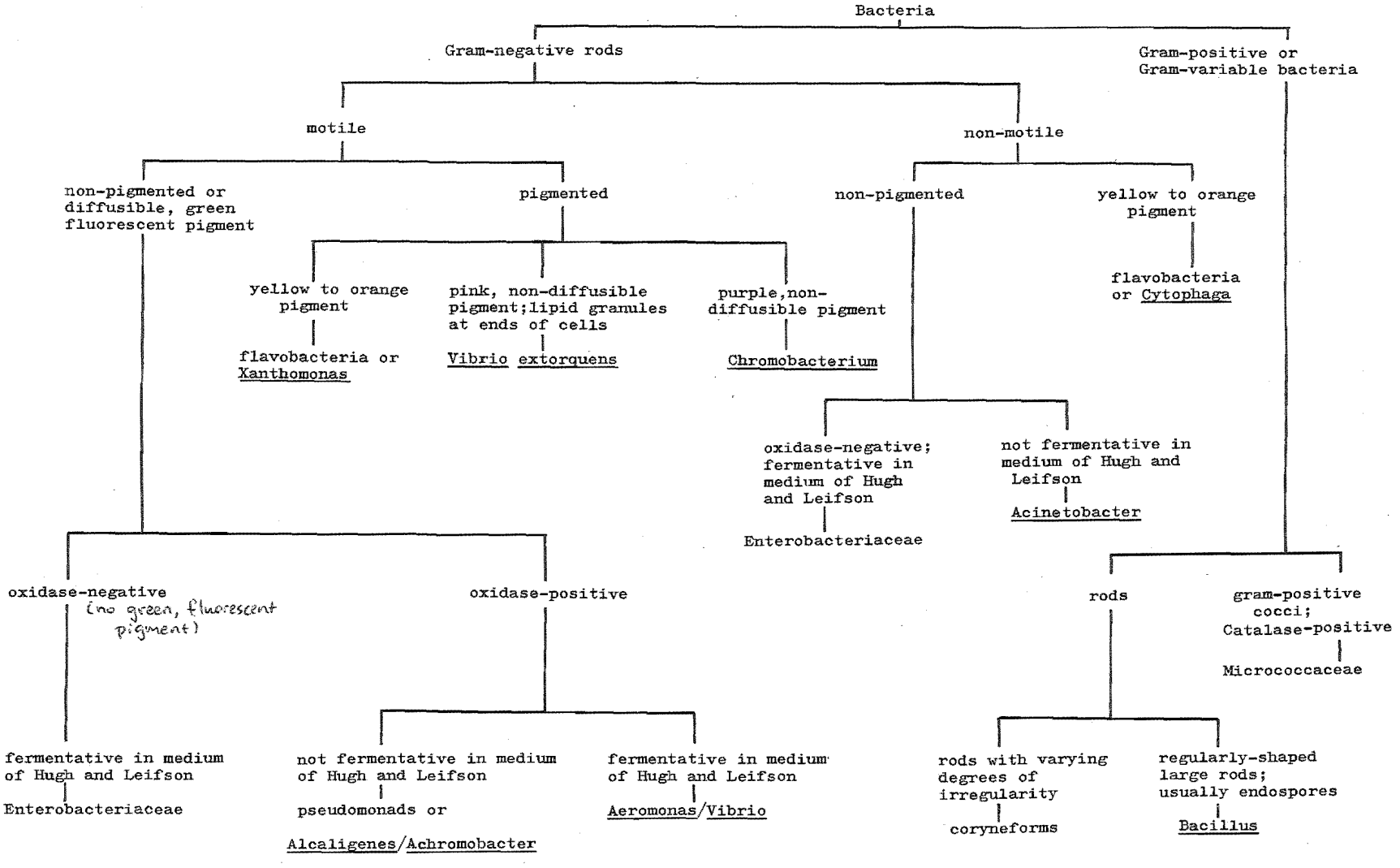
The bacteria with peritrichous flagella all had some of the following characteristics, which were not common to the polarly-flagellated bacteria:-

(i) long and irregularly-stained cells;

(ii) pleomorphic cells;

(iii) regularly-shaped cells but only oxidative in medium of Hugh and Leifson after 12 to 14 days;

TABLE 2.3 Outline of scheme used to identify bacteria from freshwater habitats



(iv) a cream-coloured colony with crinkles or whorls in it. This last kind of bacterium was distinct from a number of isolates which had very white colonies which grew abundantly on GYCA and developed crinkles giving the colony a 'curly' appearance after incubation for 4 days. These isolates had small, slender, slightly-curved cells with a single polar flagellum. Their utilization of glucose varied from oxidative to not utilized.

The characteristics (i) to (iv) were used to separate isolates with peritrichous flagella, which were initially grouped as pseudomonads, from the polarly-flagellated pseudomonads.

The ability to produce a diffusible, fluorescent, green pigment was used to differentiate Pseudomonas fluorescens from the remainder of the pseudomonads. These bacteria were also the only pseudomonads to produce ammonia from arginine. The majority utilized glucose oxidatively but a few utilized glucose fermentatively.

Also grouped as pseudomonads were a small number of bacteria which were initially keyed out into groups other than pseudomonads. However, apart from being either oxidase-negative or non-motile they had the characteristics of pseudomonads and so they were finally designated pseudomonads.

b) Vibrio extorquens

Among the pseudomonads was a distinct group of pink-pigmented bacteria. These had characteristic lipid granules usually at both ends of the cell, could utilize methanol as a sole carbon source and had a single polar flagellum. They appeared to be similar to Vibrio extorquens described by

Stocks and McCleskey (1964).

These bacteria were occasionally conspicuous in the lake and, as they were so distinctive, they were separated from the pseudomonads. This species is examined in more detail in Chapter 11.

c) Alcaligenes/Achromobacter

Bacteria which were separated from the pseudomonads because they had the characteristics (i)-(iv) mentioned in a) above, were put into the group Alcaligenes/Achromobacter. In the scheme of Shewan, Hobbs and Hodgkiss such bacteria are not included. Bacteria with peritrichous flagella which are not fermentative are, however, included in the amended scheme of Hendrie, Hodgkiss and Shewan (1964), but are described as resembling the type strain of Achromobacter - A. liquefaciens (Breed, Murray and Smith, 1957), in having 'short, rather thick rods with rounded ends...'. In the present study, bacteria assigned to the group Alcaligenes/Achromobacter did not form a homogeneous group and very few of them had short thick rods. Shewan (1963) mentions that Gram-negative bacteria with rather long rods with square ends and peritrichous flagella, which were not obviously Enterobacteriaceae, were found occasionally in the marine environment. He did not assign them to any genus.

In her review of Acinetobacter and related genera, Thornley (1967) suggested that aerobic, Gram-negative bacteria, which were non-pigmented, did not ferment sugars and had peritrichous flagella, should be put into one of the genera Alcaligenes, Achromobacter or Agrobacterium. The grouping Alcaligenes/Achromobacter was used here.

d) Chromobacterium

Gram-negative, motile rods, which produced a blue-violet, non-diffusible pigment, were designated Chromobacterium.

e) Aeromonas/Vibrio

Gram-negative, non-pigmented, motile, oxidase-positive rods, which fermented glucose, were put into one of the genera Aeromonas or Vibrio using the scheme of Shewan, Hobbs and Hodgkiss. Distinction was made between Aeromonas and Vibrio on the basis of whether the bacteria were arginine-negative or positive in Thornley's medium. However, the numbers of bacteria in both of these genera were small so in the final analysis Aeromonas/Vibrio was considered as one group.

f) Enterobacteriaceae

Bacteria were placed in the family Enterobacteriaceae according to the scheme of Shewan, Hobbs and Hodgkiss. These bacteria fermented glucose, some with the formation of gas which was caught in the semi-solid medium, within 24 h, although some isolates were not examined until after 4 days. Many grew abundantly and formed white, often mucoid, colonies on GYCA. Gram-staining showed that most of the isolates had a proportion of short rods but there were not many isolates which had only short cells. Some non-motile isolates were also included in the family. The Enterobacteriaceae are considered further in Chapter 5.

g) Acinetobacter

According to the scheme of Shewan, Hobbs and Hodgkiss,

Gram-negative, non-pigmented, non-motile bacteria, which have typically short, stout and often coccoid rods, fall into either the genus Achromobacter or Alcaligenes. In the present study these bacteria are recognized by the same basic characters used by Shewan, Hobbs and Hodgkiss, but in agreement with the designation by Thornley (1967) of Gram-negative, motile, non-fermentative rods with peritrichous flagella as either Alcaligenes, Achromobacter or Agrobacterium, they are put in the genus Acinetobacter. Hendrie, Hodgkiss and Shewan (1964) also suggested that such non-motile bacteria should be assigned to Acinetobacter.

The following additional characteristics of Acinetobacter, noted by Thornley (1967), were taken into account when grouping the bacteria:- '... catalase-positive, sugars oxidized or not at all, arginine test-negative.'.

h) Cytophaga

Gram-negative, non-motile, yellow-orange-pigmented bacteria with long slender rods were designated Cytophaga. Many of the isolates produced spreading colonies. None of those tested could degrade cellulose. More characteristics of these bacteria are described in Chapter 10.

i) flavobacteria

Hendrie, Mitchell and Shewan (1968) recommended that before relegating yellow-pigmented bacteria to Flavobacterium they should be assigned to the genera to which they were most closely related, although these might only contain a few yellow-pigmented representatives. The bacteria designated flavobacteria in this study were an extremely diverse group but as

many appeared to have similar ecological niches it was preferable not to spread them among other genera when any ecological correlation might be masked.

The flavobacteria isolated from Lake Grasmere gave varying biochemical reactions to many of the tests used. Their pigmentation ranged from yellow-green to yellow to orange. None of those tested could degrade cellulose. They were divided into two groups on the basis of motility. The non-motile bacteria were differentiated from Cytophaga by their lack of long slender rods and the absence of spreading colonies.

There was a tendency for certain results of the tests for the presence of catalase and oxidase, and the mode of utilization of glucose to correspond with one of the two groups (Table 2.4). The motile flavobacteria appeared to be more physiologically-active, with the majority being catalase-positive and many being oxidase-positive and having an oxidative utilization of glucose. Among the non-motile flavobacteria results were more variable but many failed to grow in the medium of Hugh and Leifson. However, the proportions of non-motile and motile flavobacteria which were oxidase-positive were very similar.

The two groups of flavobacteria recognized were therefore:-

(i) non-motile - rods short or medium in length but not long and slender; oxidase, catalase and mode of utilization of glucose all variable; spreading colonies not formed.

(ii) motile - oxidase, catalase and mode of utilization of glucose all variable but bacteria tend to be more physiologically-active than the non-motile group.

TABLE 2.4 Characteristics of motile and non-motile flavobacteria

Motility	No. isolates	No. of isolates giving result: Presence of catalase		Presence of oxidase		Utilization of glucose in medium of Hugh and Leifson[1]				
		+	-	+	-	alk	ox	ferm	nc	ng
Motile	99	91	8	73	26	5	64	2	23	5
Non-motile	100	57	43	73	27	6	19	1	18	56

[1] ox - oxidative; ferm - fermentative; alk - medium becomes alkaline; nc - no change in pH; ng - no growth.

These two groups are similar to those suggested by Hendrie, Mitchell and Shewan (1968) but the flavobacteria from the present study had a more variable mode of utilization of glucose and some may have had polar flagella.

Among the flavobacteria isolated during this study were 17 which gave biochemical reactions similar to Xanthomonas. However, these bacteria, and any others which might have had polar flagella and should have been grouped with the pseudomonads, could not be differentiated conclusively from the yellow-pigmented bacteria, with peritrichous flagella, on the basis of the biochemical tests carried out. The flagella of all motile isolates would have had to be examined before any further separation could be undertaken. With the time available and for the purpose of this study, it was decided that a further separation based on an examination of the flagella of all the motile yellow-pigmented isolates was not warranted.

j) Micrococcaceae

Gram-positive, catalase-positive cocci were assigned to the family Micrococcaceae. No catalase-negative cocci were found. Differentiation of genera was not attempted as cocci did not make up a major part of the lake population. Of 93 cocci isolated 75% were pigmented greenish, yellow or orange.

k) Bacillus

Gram-positive bacteria, which had regularly-shaped, large rods and usually endospores, were put in the genus Bacillus.

l) coryneforms

Gram-positive or variable bacteria which were not assigned to the genus Bacillus were grouped as coryneforms. This was a diverse group and included motile and non-motile bacteria which had rods with varying degrees of irregularity. Of the 265 coryneform isolates 64% were pigmented various shades of green, yellow, orange or red.

Some of the isolates in three of these 12 groups were characterized in more detail.

The following tests were carried out on some of the Enterobacteriaceae isolates:-

Incubation was at 37°C except where specified.

Fermentation of sugars: Filter-sterilized lactose, glucose or inositol (1%, w/v) was added to a basal medium of nutrient broth and 0.01% (w/v) phenol red (Harrigan and McCance, 1966). All bottles were examined for acid and gas formation after 1 and 4 days incubation. Some were also examined after 2

days. Fermentation of salicin was tested using Key Salicin fermentation tablets (Key Scientific Products Ltd.).

Methyl red (MR), Voges-Proskauer (VP): Glucose phosphate broth and the methods described by Harrigan and McCance (1966) were used. In most cases the tests were carried out twice - after incubation at 26°C for 2 days and after re-inoculation and incubation for 4 days at 30°C. The VP test of Barritt (1936) was used.

Citrate utilization: Simmon's citrate agar (Harrigan and McCance, 1966) was incubated at 26°C and examined after 2, 4 and 7 days.

Presence of Urease: Key Urease tablets (Key Scientific Products Ltd.) were used.

Presence of phenylalanine deaminase: The phenylalanine medium and method described by Harrigan and McCance (1966) were used.

Gelatin liquefaction: Difco gelatin (10%, w/v) was added to the Cytophaga medium described by Anderson and Ordal (1961). Stab cultures were incubated at 26°C and examined after 2, 4 and 8 days after first cooling in the refrigerator.

H_2S production: Stab cultures in Kliger's iron agar (Key Scientific Products Ltd.) were incubated at 26°C and observed after 18 h, 2 and 4 days.

Fermentation of lactose in McConkey broth: Difco Bacto McConkey broth was inoculated with a 24 h nutrient broth culture which had been incubated at 30°C. Bottles were incubated at 30°C and examined daily for 4 days.

The following tests were carried out on some of the Cytophaga isolates:

Incubation was at 20°C.

Presence of phosphatase: Phenolphthalein diphosphate (0.01%, w/v) was incorporated into nutrient agar. Plates were streaked with the test organism and after incubation for 3-5 days, were exposed to ammonia vapour (Hendrie, Mitchell and Shewan, 1968).

Sensitivity to polymyxin B: Test cultures were spread on the surface of nutrient agar plates and polymyxin B sensitivity discs (Oxoid, 300 units) were placed on the plates. Plates were examined for any inhibition zones after incubation for 3-5 days (Hendrie, Mitchell and Shewan, 1968).

Cellulose degradation:

(i) Cellulose acetate (3%) and agar (1%) were added to the Cytophaga medium of Anderson and Ordal (1961). Plates were inoculated and after incubation for 3-10 days were examined for clearing of the medium.

(ii) Filter paper was added as a source of cellulose to a fermentation broth made up of peptone - 0.15, yeast extract - 0.05, beef extract - 0.05 (%,w/v) (Anderson and Ordal, 1961). Bottles were incubated for 5 weeks and examined for evidence of degradation of the filter paper.

Cell wall stain: Cells were stained with phosphomolybdic acid as described by Hale (1953).

Presence of microcysts: Isolates were subcultured to Cytophaga medium (Anderson and Ordal, 1961), designated AO medium here, with 0.4% agar added. After incubating for 5 days cells were examined with phase-contrast illumination for microcysts.

Unless, otherwise stated the following tests were carried

out as described by Lewin and Lounsbery (1969) except that AO medium was substituted for Medium 2 of Lewin and Lounsbery and the standard incubation temperature was 20°C not 30°C.

Survival at +3°C

$urvival at -196°C

Lauryl sulphate tolerance

Tyrosine degradation

Nitrate reduction

Starch hydrolysis

Agar liquefaction: Incubation was at 30°C and was extended to 8 days.

Alginate liquefaction: Sodium alginate was used and incubation was extended to 14 days.

Gelatin liquefaction: Cultures were examined after 3 and 7 days.

Catalase production: Plate cultures were used.

Reaction in litmus milk medium: Difco Bacto litmus milk medium was used and sterilized by autoclaving for 15 min at 15 lbs pressure.

Presence of oxidase: Cultures on AO agar were tested for the presence of oxidase using the method of Kovacs (1956).

Hydrogen sulphide production: Cystine (0.01%, w/v) was added to AO broth. Lead acetate papers, prepared as described by Harrigan and McCance (1966), were inserted over inoculated broths. Cultures were examined for any blackening of the paper after incubation for 3-7 days.

Salinity tolerance: The salinity tolerance of isolates was tested by incorporating different concentrations of sodium chloride (0,14,28 and 56 g/l) into AO agar. The salinity of sea water was assumed to be 28 g/l (Jannasch and Jones,

1959). Inoculated plates were incubated for 2-7 days and examined for evidence of growth or inhibition.

Penicillin tolerance:

(i) Plates of AO agar were poured with penicillin (Benzyl penicillin (sodium salt), Glaxo Labs. N.Z. Ltd.) added after autoclaving to give the following final concentrations (units/ml): 10^{-1}, 10^{-3}, 10^{-5}, 10^{-6}, 10^{-7}. Three to seven days after inoculation, plates were examined for evidence of growth or inhibition.

(ii) Plates were spread inoculated with test cultures and sterile filter paper discs were placed on the plates. Aseptic dilutions of penicillin were prepared and 0.1 ml of each dilution was placed on the discs to give the following range of dilutions (no. units/disc) : 10^{3}, 10^{2}, 10, 10^{-1}, 10^{-3}, 10^{-5}, 10^{-7}. After incubating for 4-7 days, plates were examined for inhibition zones.

Temperatures limits for growth: Broth cultures of Medium 2 of Lewin and Lounsbery, with sodium glycerophosphate (0.1 g/1) replaced by Na_2HPO_4 (0.1 g/1) and glycerol (0.5 ml/1), were incubated at 30, 35 and 40°C and examined for turbidity for up to 7 days.

Effect of temperature on growth rate: Isolates were inoculated into five replicates of Medium 2 of Lewin and Lounsbery (1969) modified as described above. Replicates were incubated at 13, 20, 26, 30 and 35°C. Duplicate bottles were set up for three of the isolates incubated at 26°C. Samples were taken at intervals for up to 7 days and their turbidity measured with a colorimeter (Evan Electroselenium Ltd.), using a green filter.

Methyl cellulose degradation: Three per cent methyl cellulose was added to A0 medium and sterilized by autoclaving. Stab cultures were incubated for 14 days and examined for liquefaction.

Nitrogen source: The basal medium was Medium 2 of Lewin and Lounsbery (1969), modified as described above and without KNO_3 or casamino acids. One of the following nitrogen sources was added (1 g/1) : tryptone, casamino acids, sodium glutamate, potassium nitrate. Each isolate was inoculated into a set of four bottles containing the different nitrogen sources and also into a control which contained no added source of nitrogen. Growth after 2-5 days was compared with the control. Any isolates giving doubtful results were reinoculated from the control tube into fresh test media.

Carbon source: A basal medium of Medium 1 using distilled water not sea water, was prepared with sodium glycerophosphate replaced by Na_2HPO_4 and glycerol as above, and with tryptone and yeast extract each at 0.2 g/1 instead of the higher concentrations recommended. The method and carbon sources of Lewin and Lounsbery (1969) were used to determine which carbon sources were suitable for growth.

Pigmentation: An emulsion always formed when the methods of Lewin and Lounsbery (1969) or Fox and Lewin (1963) were used to extract the pigments into ethanol and transfer them to petrol ether. A modification of the two methods was therefore used to obtain an extract in petrol ether (boiling point range 40-60°C).

Flasks containing 200 ml of A0 broth were inoculated and after incubating in the dark for 4 days, were harvested by

centrifuging for 12 min at 6 500 g. The concentrated cells were resuspended in ethanol and left in a refrigerator for several days to extract the pigment. Extracts were filtered through a 0.5-1 cm layer of celite (B.D.H. 80-120 mesh) on a sintered-glass filter. The absorption spectrum of the resulting clear orange pigment solution in ethanol was examined with a Beckman DB spectrophotometer. The ethanolic solution was then concentrated by boiling to about a quarter of the original volume and run on to a bed of celite on a sintered-glass filter. The small volume of ethanol (about 5-7 ml) was carefully drawn off by vacuum when the pigment remained on the filter. The pigment was extracted with petrol ether and the absorption spectrum examined in the spectrophotometer.

The following tests were carried out on some of the Vibrio extorquens isolates:-

Staining for spores:

(i) Bartholomew and Mittwer's "Cold" Method (Manual of Microbiological Methods, 1957).

(ii) Dorner's Method - Snyder's Modification (ibid).

Staining for fat droplets: Burdon's Method (ibid).

Utilization of methanol as a sole carbon source:

(i) The medium of Kaneda and Roxburgh (1959) was prepared with 1.5% agar. Plates were poured with and without methanol (0.5%, v/v) added after autoclaving. Isolates of Vibrio extorquens, which had been growing on a mineral salts agar in which the only carbon source was autoclaved methanol, were streaked on to plates of methanol medium, control plates of GYCA and control plates of the medium of Kaneda and Roxburgh

(1959) without methanol. Incubation was at 20°C for 6 days.

(ii) Some isolates were also tested in the liquid mineral salts medium of Stocks and McClesky (1964) with 0.5% (v/v) methanol added after autoclaving. The methanol was filtered through a sintered-glass filter before adding to the medium. Controls without methanol were included. Incubation was at 20°C.

2.5 ENRICHMENT CULTURES

Enrichment for cellulose-degrading bacteria

One ml of water or concentrated plankton, or the first dilution of Elodea or mud was added to 4 ml of mineral salts medium, No. 2, with filter paper as the source of cellulose (Harrigan and McCance, 1966). Bottles were incubated at 20°C and examined for up to 14 days. If the filter paper had been degraded, samples were streaked on to GYCA. Possible cellulose-degarding isolates were reinoculated into bottles of the cellulose mineral salts medium to test the cellulose-degrading ability of the pure cultures.

Enrichment for methanol-utilizing bacteria

Inocula, as described above, were added to 10 to 15 ml of the medium of Stocks and McCleskey (1964) with 0.5% (v/v) methanol added. Incubation was at 20°C for 2 to 3 weeks. Samples from bottles showing turbidity were streaked on to GYCA and in some cases colonies were isolated and inoculated into fresh methanol medium. On one sampling date bottles were duplicated and incubated at 20°C and 30°C.

2.6 DIRECT ENUMERATION OF BACTERIA

Direct counts were made of bacteria in water samples and on Elodea leaves. The direct microscopic method provides a count of all bacteria visible, whether alive or dead. The main problem of this method is distinguishing bacteria from other small particles and this was particularly serious with the water samples.

Lake water

Bacteria were concentrated by filtering 5 to 20 ml of lake water, which had been fixed with Lugol's iodine within 7 h of sampling, through a membrane filter (pore-size, 0.45 μm; diameter, 13 mm), using a Swinny filter holder (Millipore Filter Corp.). The filter was stained with 0.3% (w/v) aqueous methylene blue (Meynell and Meynell, 1970) for 5 min by placing the filter on a glass slide and putting a drop of stain on top of the filter. Excess stain was removed with water and, when dry, the filter was cleared with either immersion oil (refractive index 1.515), when the visibility was poor, or with dimethyl sulphoxide in isopropanol as described in the Gelman Instrument Co. Product Bulletin 221c (1967). The latter method of clearing made the filters more transparent, but the clumping of detritus, the presence of many algae compared to the numbers of bacteria, and the small size of the bacteria all made it hard to distinguish bacteria.

When a 10 to 15 ml sample was macerated with an MSE homogenizer, the sample was more homogeneous but this method was not very satisfactory as bacteria were detached from

particles and organisms in the sample thus increasing the count of bacteria free in the water. Because bacteria were more easily resolved on a glass slide than on a filter, an approximation of the number of bacteria in one sample was obtained by transferring a filtered, macerated sample to a marked square on a glass slide.

An eyepiece with a rectangular grid was used to count the bacteria. The numbers of bacteria within the rectangle in 50 microscope fields were recorded and from these data the numbers of bacteria per ml of water were calculated.

Elodea canadensis

An estimate of the numbers of bacteria on Elodea leaves, which had been fixed in Lugol's iodine, was obtained by macerating 2 to 3 leaves in 10 ml of filter-sterilized water. The macerated leaf suspension was diluted so that, when 5 to 7.5 ml was concentrated by filtering, as described above, there was a suitable concentration of bacteria for counting. The filter was stained with methylene blue and cleared with dimethyl sulphoxide in isopropanol. While the bacteria in these samples were small, they were much more easily distinguished than the bacteria in the water samples. This was mainly because there was less detritus and fewer organisms such as algae masking the bacteria.

The number of bacteria on the filter was determined by counting the numbers of bacteria within the rectangular grid in 50 to 108 fields across a diameter of the filter. Counts of bacteria were expressed as the number of bacteria per mm^2 of leaf surface. The surface area of the leaves was estimated from an additional sample of three leaves as des-

cribed on p.24.

2.7 ENUMERATION OF ALGAE

Lake water

Samples of lake water (5-10 ml), fixed with Lugol's iodine, were filtered through Millipore filters (pore-size, 0.45 μm; diameter, 13 mm). After staining with methylene blue and clearing with dimethyl sulphoxide in isopropanol, the numbers of algal cells in each field across a diameter of the filter were counted using an oil immersion objective. The results were expressed as the number of algal cells per ml of lake water.

Elodea canadensis

In one experiment the numbers of epiphytic algae of the genus Cocconeis on Elodea leaves were counted. Leaves, which had been fixed in Lugol's iodine, were mounted in water under a coverslip and examined at a magnification of x 215. The numbers of algae per microscope field were counted for various parts of the upper and lower surfaces of leaves. Results were expressed as percentage cover of algae per microscope field. The mean area covered by one algal cell was determined from measurements of 10 cells on one leaf, assuming the shape of Cocconeis was an ellipse. This area was $119 \pm 21\ \mu m^2$.

2.8 ENUMERATION OF ZOOPLANKTON

The zooplankton in lake water samples (1 or 2 l), which had been passed through a No. 15 net (mesh-size, 94 μm), were

fixed with a formalin/alcohol mixture (5% formalin, 35% alcohol, 60% water). A count of all the animals present was made using Sedgwick-Rafter counting cells. The results were expressed as the number of animals per litre of lake water.

2.9 CULTURE OF ALGAE

Diatoms from Lake Grasmere were isolated and eventually one species was obtained as a 'nearly bacteria-free' culture. This alga was used in experiments designed to examine the interactions between bacteria and algae. Sample bottles were only half-filled to ensure sufficient oxygen for the algae and the samples were kept in an iced container until they were treated in the laboratory within 5 h of sampling.

Enrichment cultures

Enrichment cultures were set up by adding 1 ml of inoculum to 50 ml of medium in pyrex conical flasks. The inoculum was either untreated lake water or lake water which had been passed through a No. 15 net to concentrate the algae.

The medium used for all enrichment cultures and most of the subsequent culturing was a modification of Medium no. 10 of Chu (1942) and is given in Table 2.5. It was identical to that described by Hughes and Lund (1962) except that 1% (v/v) soil extract (Pramer and Schmidt, 1965) was added. Medium no. 14 (modified) of Chu (1942) was used to culture a Nitzschia species (Table 2.5). If solid media were required, 1.5% agar was added.

TABLE 2.5 Algal culture media

	Quantities in mg/l glass distilled water	
	(a)	(b)
$Ca(NO_3)_2$	40	40
KH_2PO_4	5	2
$MgSO_4.7H_2O$	25	40
Na_2CO_3	20	
$CaCO_3$		10
$NaHCO_3$		40
KCl		40
Na_2SiO_3	25	25
Fe EDTA	6	6
Soil extract	10 ml/l	

(a) - modification of Chu 10; (b) - modification of Chu 14.

Cultures incubated at 12 to 17°C with 16 h light and 8 h dark. The light was provided by two fluorescent tubes (1.52 m, 80 W, white or warm white tubes) and one 15 W incandescent bulb. These had a maximum intensity of 2280 lx. After incubating for 2 to 3 weeks, algal growth was visible in the enrichment cultures.

Uni-algal and axenic cultures of algae

Two methods for obtaining axenic cultures of algae from the enrichment cultures were tried. Individual cells or small groups of cells were transferred to liquid medium, or a loop of the enrichment culture was streaked on to solid

medium and algae isolated from the colonies produced. Uni-algal cultures were obtained when groups of 5 to 10 cells were washed several times in sterile media before transferring to broth culture, but they always had a large bacterial population. Streaking the algae on to agar plates before isolating individual cells proved to be the better way of reducing the level of bacterial contamination in the algal cultures.

The following diatoms from the lake were cultured:-

a) Melosira granulata (Ehr.) Ralfs var. angustissima Müll.

This alga grew in enrichment culture but was not successfully subcultured.

b) Nitzschia sp. of Lanceolatae group

This motile diatom grew vigorously once established on plates of both the media (Table 2.5) but sometimes subcultures failed to grow. Although small groups of cells which had migrated away from the main colony and appeared bacteria-free were isolated, viable subcultures always contained many bacteria.

c) Diatoma elongatum (Lyngb.) Agardh

This species was the most conspicuous on the agar plates and after incubation for 4 weeks ribbons of cells were macroscopically visible. After 6 weeks, ribbons of cells of two lengths could be distinguished. Cells of both sizes were isolated from the ends of ribbons and were denoted 'longer taxon' and 'shorter taxon'.

The dimensions of these cells after culturing for up to 8 months are compared with those of algae in the original lake water sample in Table 2.6. The 'longer taxon' had cells a little shorter than those found in the lake sample. This

reduction in length was probably the result of continued cell division during culture. This alga grew vigorously in broth culture but the cells were always aggregated in long zigzags and not the typical 'stars' of the wild form. Even after repeated streaking on to agar and re-isolating, the 'longer taxon' had a considerable bacterial population.

TABLE 2.6 Dimensions of Diatoma elongatum cells from a field sample and from cultures of cells isolated from this sample

Material	Culture period (months)	Dimensions (μm)[1]	
		length	width (girdle view)
Field sample - 4/71	0	94±10	2.3
Longer taxon	1.25	78± 3	3.5
Longer taxon	6	79± 2.6[2]	NM[3]
Shorter taxon	5	38± 1.1	4.2-4.6
Shorter taxon (longer cells of culture)	7	34± 3.3	NM
Shorter taxon (shorter cells of culture)	7	17± 2.2	NM

[1] Mean and standard deviation.

[2] 4 cells were measured; for the other samples the lengths of at least 8 cells were measured; the width of 1 to 3 cells was recorded.

[3] NM - not measured.

The cells of the 'shorter taxon' were much shorter than those measured from the field sample (Table 2.6) and after 7 months in culture some very short cells appeared (Fig. 2.4). The reduction in length of this alga may have been artificially hastened by selecting a shorter alga from the original agar plate. The very short cells were still typically Diatoma elongatum (Lyngb.) Agardh so it is unlikely that this 'shorter taxon' was a variety of D. elongatum.

The mode of growth of the 'shorter taxon' was similar to the wild form, and, in liquid culture, it formed stellate colonies with only a few zigzag chains. After subculturing on to agar and then into liquid media an apparently bacteria-free culture was obtained. No bacteria grew when a loop of culture was transferred to nutrient broth, nutrient agar or GYCA. No bacteria were seen when a drop of culture, stained with methylene blue, was examined under the microscope. These tests were carried out 5 months after the initial enrichment culture was set up.

However, the bacteria-free cultures did not grow vigorously and on subculturing only two of six subcultures grew. Microscopic examination of one of the viable cultures showed that it was no longer axenic but had a small bacterial contaminant. As the medium might have been unsuitable to sustain bacteria-free cultures and as further investigation of this point would have been time-consuming, the cultures obtained of healthy algae of the 'shorter taxon' of Diatoma elongatum were used in the later experiments. The bacterial population of the culture was small and no bacteria were seen attached to any of a range of healthy and dead algae examined. The culture was denoted therefore as 'nearly bacteria-free'.

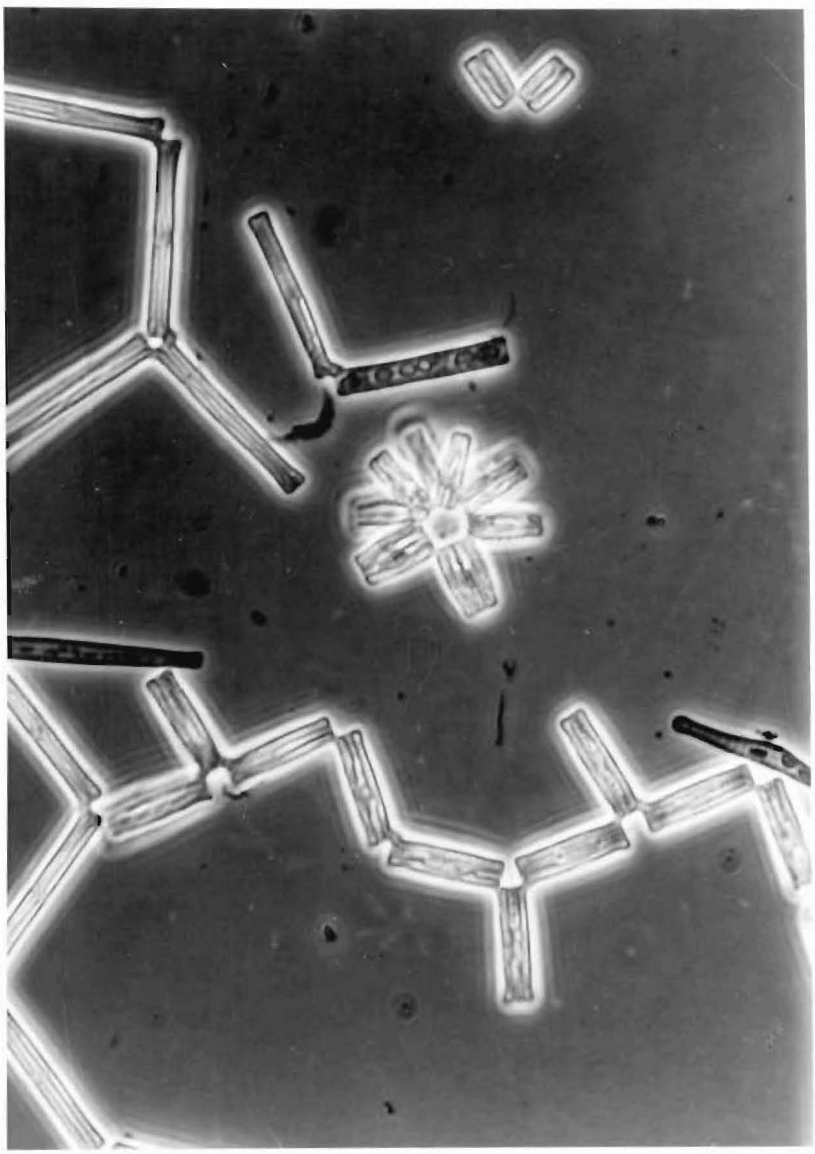


FIG. 2.4 Shorter taxon of Diatoma elongatum after 7 months in culture, showing range of cell sizes; x 720.

2.10 AUTORADIOGRAPHY

Autoradiography can be an extremely valuable method for studying the uptake of substrates by individual cells. Bacteria are incubated with a radioactive compound. A smear of the bacteria is then prepared and coated with photographic emulsion. After a suitable exposure period, the cells which have accumulated the label will produce an autoradiogram. By adjusting the concentration of isotope and time the bacteria are labelled, it is possible to obtain an autoradiogram in which only bacteria which were metabolizing at the time of sampling become labelled (Brock and Brock, 1968). If the labelled substrate is a metabolite which will be assimilated by the majority of bacteria, provided they are metabolizing, then a count of the proportions of labelled and unlabelled cells should give a measure of the metabolic activity of the population.

In this work autoradiography was used to determine the metabolic activity *in situ* of bacterial populations from various habitats within the lake. The majority of the autoradiograms were prepared for the light microscope but some methods of preparing autoradiograms for an electron microscope were investigated. Before the technique was applied to field samples, a number of experiments were carried out with pure cultures of bacteria to determine the optimal conditions for autoradiography of tritium-labelled bacteria, such that all bacteria metabolizing at the time of sampling would be detected. These conditions were used in the later field experiments. Several methods of preparing smears of field material for autoradiography were tested.

Pure culture studies

a) Treatments and methods used

The effects of varying the following factors on the detection of labelled bacteria by autoradiography were examined using pure cultures of bacteria:
(i) isotope concentration, (ii) time of incubation of the sample with the isotope, and (iii) time the emulsion was exposed to the isotope.

The following bacteria from the Canterbury University, Botany Department Culture Collection (B.D.C.C.) or isolated from Lake Grasmere (L.G.) were used:-
Escherichia coli - B.D.C.C. no. B 22;
Enterobacteriaceae - L.G. no. 6D3 4;
Pseudomonas fluorescens - B.D.C.C. no. B 5;
P. fluorescens - L.G. no. 6D3 7;
non-fluorescent pseudomonad - L.G. no. 6W 5;
Cytophaga spp. - L.G. nos. 12W 3, 13D3 1, 13D3 2.

Bacteria were incubated at 20°C for 18 - 24 h in nutrient broth (Oxoid CM1) or Cytophaga medium (Anderson and Ordal, 1961) before labelling. 0.5 - 1 ml of broth culture was added to an equal volume of isotope solution, in a bijou bottle, to give a final concentration of isotope between 1 and 20 μCi/ml. The isotope solutions were prepared aseptically with sterile distilled water. In one experiment cells from a nutrient agar slant were suspended in a small volume of sterile distilled water and were then diluted with isotope solution to give the required concentration.

The following isotopes were used:-

D-glucose-1-^{3}H: specific activities 792 mCi/m mol (used for isotope concentrations 10 μCi/ml and less) and 250 mCi/m mol (17 and 20 μCi/ml);

Thymidine (methyl-^{3}H): specific activities 17.4 Ci/m mol (10 μCi/ml and less) and 19.5 Ci/m mol (17 and 20 μCi/ml);

Tritiated water: specific activity 5 mCi/ml.

Bacteria were incubated with the label for 1-2 h at room temperature and then fixed with Lugol's iodine. Controls in which bacteria were fixed before labelling or were not labelled were included. In one experiment, bacteria were fixed with 3% glutaraldehyde in phosphate buffer, pH 7.

The results of six experiments (Table 2.7) are described in this section. For each experiment the bacteria had originated from one broth culture or agar slant. A single autoradiogram was prepared for each treatment. Smears of bacteria were made at one end of slides which had previously been cleaned, and then subbed with gelatin (Gude, 1968). After heat fixing, the smears were washed in a beaker of running water for 30 s and air dried. They were then ready to be dipped in emulsion.

Ilford liquid nuclear emulsions were used in all experiments. These are made in three grain sizes, G, K and L (larger to smaller), each with a range of sensitivities 0-5. Only emulsions of sensitivities 4 and 5 will competently detect higher energy isotopes such as ^{14}C, but level 2 is sufficient to detect tritium β radiation. In the pure culture experiments K2 emulsion was used predominantly. The early field experiments were also carried out with K2 emulsion but, for the later field experiments, L4 emulsion

TABLE 2.7 Treatments used in autoradiographic experiments with pure cultures of bacteria

Exp.	No. isolates tested	Isotope[1]	Isotope conc. (μCi/ml)	Incubation period (h)	Exposure period (days)	Results shown in
I	2	Glu	1	1	3, 4	Text p. 68
II	3	Tdr, Glu	5, 10	1	3, 7, 14	Fig.2.5
III	3	Glu	20	1, 2	7, 14	Fig.2.5
IV	3	Tdr	20	1, 2	7, 14	Fig.2.5
V	2	Tdr, Glu	17	1.75	7	Table 2.8
	1	Glu	17	1.75	3	
VI	1	Glu	20	2	7	Table 2.8

[1] Glu- ^{3}H-glucose; Tdr - ^{3}H-thymidine.

was used.

The method of diluting both emulsions was the same. In the darkroom, with orange (Ilford 9025) and red (Kodak 1A) safelights, the emulsion was melted in a flattened test tube (Gude, 1968), in a covered waterbath at 43°C for 1 h. The test tube was stood in a small screw-cap jar inside a light-tight film container. After melting, the emulsion was diluted with an equal volume of distilled water heated to 43°C. Slides were dipped singly and the emulsion wiped off the back. They were placed on a cold steel strip until the emulsion had gelled (Rogers, 1967) and then put in a glass slide rack. These racks were placed with a silica gel bag in a light-tight container (B.T.L. anaerobic jar) with metal stands between the racks, so that up to 30 slides could be put in each jar.

After allowing the slides to dry at room temperature for 2 h, they were exposed in the jars at 4°C for 3-14 days. Blank slides were included in each experiment to assess the background level of grains.

Photographic processing was carried out in glass staining dishes at 20°C, as follows:-

	K2	L4
Developer	ID-11 (10 min)	ID-2 diluted 1:1 (4 min)
Stop	tap water (10 s)	1% acetic acid (10 s)
Fixative	20% (w/v) sodium thiosulphate (7 min)	May and Baker Super Amfix 1:6 (5 min)
Wash	tap water changed 5 times over 12 min	tap water changed 5 times over 10 min

The slides were air dried, stained with 0.3% (w/v) aqueous methylene blue for 30 s and examined using bright field optics. Immersion oil was placed directly on the film and the percentage of labelled cells was counted. No attempt was made to count individual grains.

b) Statistics

The formula $n = \frac{4pq}{L^2}$, where n is the sample size, p and q are the percentages of labelled and unlabelled cells, respectively, and L is the allowable percentage error, which for this work was set at 5%, was used to determine the number of cells to be counted (Snedecor and Cochran, 1967). Because n, calculated from this formula, increases as p tends to 50%, an estimate of the sample size required for each slide was

made after 100 cells had been counted.

In most cases the percentage of labelled cells was calculated from a single count. With the sample sizes taken these percentages should not deviate by more than ± 5% from the true value within confidence limits of 95%, provided the sample is distributed binomially on the slide. To ensure that the labelled bacteria were evenly distributed on the slide, replicate counts from three areas of the slide were made for three of the treatments which had different levels of labelled cells. The standard deviations of these counts were very small, viz. 92 ± 1; 31 ± 2; 58 ± 2 (% labelled cells).

c) Concentration of substrate and length of incubation period

If this technique is to be used to detect only those bacteria metabolizing at the time of sampling, the conditions of incubation must not result in the growth of inactive bacteria. For this reason, concentrations of substrate were kept as low as possible (glucose up to 14.3 mg/l, thymidine (Tdr) up to 0.248 mg/l) by using isotopes with high specific activities, and samples were incubated for not longer than 2 h. Before labelling, bacteria were cultured in media which did not contain either glucose or Tdr so that the addition of cells and medium to the isotopes would not reduce their specific activities. It was thought that having a rich nutrient medium, such as nutrient broth, although diluted by half, in the incubation mixture might result in cells less likely to metabolize low levels of glucose and Tdr. Therefore, for the experiments with the higher isotope concentrations (17 and 20 μCi/ml),

either the more dilute Cytophaga medium or cells from nutrient agar slants were used.

d) Conditions ensuring optimal detection of labelling by autoradiography

In these experiments bacteria were initially cultured under favourable conditions so that when they were labelled nearly all the cells would have been metabolizing. The aim was therefore to obtain autoradiograms in which nearly all the cells were labelled as in these autoradiograms detection of labelling would be optimal.

The use of tritiated water as a label was not satisfactory because the specific activity of the isotope was reduced too much when it was diluted to give the required level of radioactivity. With ^{3}H-water concentrations up to 29 μCi/ml, no accumulation by an Enterobacteriaceae isolate of tritium label could be detected after exposure for 14 days.

A number of the early experiments were carried out using an isotope concentration of 1 μCi/ml as recommended by Brock and Brock (1968). However, no uptake of the isotope was detected. For instance, two lake isolates (Enterobacteriaceae and Cytophaga) which had been labelled with 1 μCi/ml of ^{3}H-glucose for 1 h failed to produce an autoradiogram after exposure for 3-4 days. The lack of detection of labelling could have been due to the large number of metabolizing cells present. Each cell may have accumulated only a very small amount of isotope. Recently, Paerl and Goldman (1972) have reported obtaining autoradiograms of ^{14}C-acetate-labelled bacteria from oligotrophic Lake Tahoe after incubation for 1 h and exposure for 3 to 5 days. Water was

labelled immediately after sampling with a final concentration of acetate of 0.05 μg/l. The specific activity of the ^{14}C-acetate reported was 50 μCi/μ mol so that the isotope concentration was only about 40 pCi/ml.

The effect of higher concentrations of isotope and different incubation and exposure periods on the percentage of labelled bacteria detected by autoradiography was studied. The results are shown in Figure 2.5. The percentage of labelled cells of three kinds of bacteria, each incubated with ^{3}H-glucose and ^{3}H-Tdr, has been plotted against increasing exposure time. For the isotope concentration of 20 μCi/ml the effect of 1 and 2 h incubation is shown. With 5 and 10 μCi/ml of both isotopes, an increasing number of cells had silver grains over them with the extension of exposure time but, except for the labelling of Escherichia coli with 10 μCi/ml ^{3}H-glucose, 20 μCi/ml of both compounds and 14 days exposure were needed to ensure that all metabolically-active cells were detected (Fig. 2.5).

The results of labelling pseudomonad 6W 5 with ^{3}H-Tdr and Pseudomonas fluorescens L.G. with ^{3}H-glucose (Table 2.8) showed that when an isotope concentration of 17 μCi/ml was combined with 1.75 h incubation and 7 days exposure, not all cells capable of metabolizing the substrate formed an autoradiogram. The labelling of P. fluorescens L.G. with ^{3}H-glucose (Table 2.8) can be compared with the number of labelled cells in the autoradiogram of P. fluorescens B.D.C.C. labelled with 20 μCi/ml ^{3}H-glucose, incubated for 2 h and exposed for 7 days (Fig. 2.5). When an autoradiogram of P. fluorescens L.G. was prepared with a lower ^{3}H-glucose concentration and

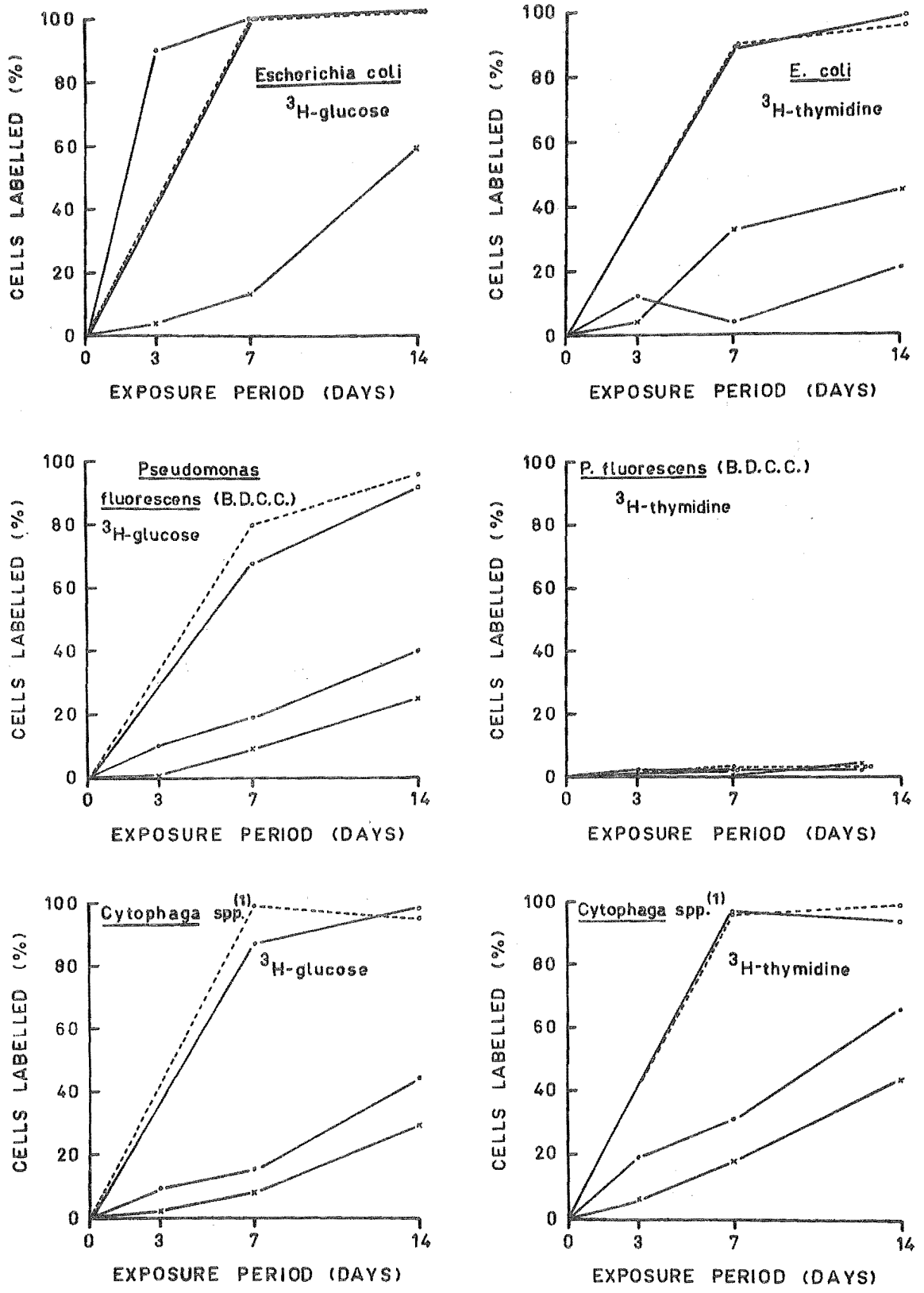


FIG. 2.5 The effect of different incubation and exposure periods, and concentrations of isotope, on the percentage of labelled bacteria detected by autoradiography.

× 5 μCi/ml; ∘ 10 μCi/ml; ∘ 20 μCi/ml; —— 1 h incubation; ------ 2 h incubation.

(1) Cytophaga no. 13D3 2 - 20 μCi/ml; no. 13D3 1 - 5 and 10 μCi/ml

TABLE 2.8 Detection of uptake of ^{3}H-glucose and ^{3}H-thymidine by two pseudomonads using autoradiography

Isolate[1]	Isotope	Concentration of isotope (μCi/ml)	Incubation period (h)	Exposure period (days)	Bacteria labelled (%)
pseudomonad 6W 5	Glu	17	1.75	7	94
pseudomonad 6W 5	Glu	17	1.75	3	90[2]
pseudomonad 6W 5	Glu	20	2	7	92[3]
pseudomonad 6W 5	Tdr	17	1.75	7	63
P. fluorescens L.G.	Glu	17	1.75	7	66
P. fluorescens L.G.	Tdr	17	1.75	7	7

1 Both isolates were grown on nutrient agar slants before labelling.

2 L4 emulsion, iodine fixation.

3 L4 emulsion, glutaraldehyde fixation; in remainder of treatments bacteria were fixed with Lugol's iodine and K2 emulsion was used.

slightly shorter incubation period, fewer labelled cells were detected. However, for both P. fluorescens isolates uptake of ^{3}H-Tdr was almost undetectable (Fig. 2.5; Table 2.8). This is discussed on pp. 73-76.

Thus, for the field experiments an isotope concentration of 20 μCi/ml was used with an exposure period of 14 days and an incubation period of 1.75-2 h. Although the shorter incubation period of 1 h produced the same percentage of labelled cells as 2 h incubation, after 14 days exposure (Fig. 2.5), the longer incubation period would be more likely

to allow weakly metabolizing cells, which might be found in natural habitats, to incorporate detectable amounts of isotopes.

Table 2.8 also includes results obtained with L4 emulsion over glutaraldehyde- and iodine-fixed bacteria. Although the treatments were not identical, varying the fixative made little difference to the percentage of labelled cells in the autoradiograms of pseudomonad 6W 5 and, furthermore, similar percentages of labelled cells were obtained using K2 and L4 emulsions.

e) Suitability of glucose and thymidine as a substrate

In this type of experiment, where the metabolic activity of a mixed population of bacteria is to be estimated by the uptake of one or two substrates, there is the problem that not all actively-growing bacteria will be able to metabolize the substrate provided. The cell may not be permeable to certain compounds or may lack enzymes to utilize them.

Most heterotrophic bacteria are permeable to glucose and have suitable enzymes to utilize it. Therefore, this carbohydrate was chosen to provide a general measure of the metabolic activity of the bacterial population. Neglecting those bacteria that cannot take up glucose will introduce only a small error, providing autotrophic bacteria do not make up a significant part of the population.

By scoring cells as labelled or unlabelled, an indication of the proportion of the population metabolizing at the time of sampling is obtained. However, counts of the percentage of ^{3}H-glucose-labelled cells will not indicate how active the cells in the labelled group are. Grain counts would show

how much label had been accumulated within individual bacteria, but these might well not be directly related to the activity of a bacterium to the range of substrates available in its natural environment. A low grain count might be indicative of slow uptake of ^{3}H-glucose because, either metabolic activity as a whole was very slow, or the organism might not be adapted to glucose uptake at that time, but be metabolizing other compounds vigorously.

One way to determine the level of metabolic activity among the active bacteria is to study the rate of cell division through uptake of ^{3}H-Tdr. Thymidine is incorporated into DNA, which is synthesized only when cell division is about to take place. In slowly dividing bacteria, there is a period when the cell is not synthesizing DNA. This period shortens as the rate of cell division increases until, with rapidly dividing cells, DNA synthesis is continuous (Pritchard, Barth and Collins, 1969). Therefore, determining the number of cells taking up Tdr provides an estimate of the proportion of the population that is about to divide.

However, interpreting ^{3}H-Tdr-uptake data is complicated because, in several kinds of bacteria, Tdr is not incorporated into DNA. Of the five pure cultures of bacteria examined in this study, two, which were strains of one species, did not take up Tdr.

The lack of uptake of Tdr may be due to:

(i) active degradation of Tdr to thymine by enzyme Tdr phosphorylase (EC2.4.2.4.). Exogenous thymine is not readily incorporated into DNA;

(ii) lack of Tdr kinase enzyme (EC2.7.1.21) to incorporate Tdr into DNA;

(iii) the cell is not permeable to the compound (Grivell and Jackson, 1968).

Rachmeler, Gerhart and Rosner (1961) have shown that situation (i) exists in certain strains of Escherichia coli which incorporate only a small proportion of Tdr supplied into DNA. Tdr phosphorylase is induced and there is a consequent degradation of Tdr to thymine. Until all the exogenous Tdr has been converted to thymine, some uptake of Tdr will however take place.

In the field, with mixed bacterial populations, the importance of this enzyme system would depend on the numbers of bacteria possessing it and the rate of induction of the phosphorylase. In the 1.75-2 h incubation periods of the field autoradiographic experiments some degradation of Tdr may not significantly reduce the uptake of Tdr into cells but the possibility that some Tdr is being degraded should be noted when interpreting results.

Situation (ii), where Tdr is not incorporated into DNA, is more serious as cells without a kinase enzyme will never have labelled DNA. Tdr kinase has not been detected in cell-free extracts of Neurospora crassa, Aspergillus nidulans, Saccharomyces cerevisiae or Euglena gracilis (Grivell and Jackson, 1968), but the lack of this enzyme system in any bacteria has not been positively demonstrated.

There is, however, some work which suggests that Tdr kinase may be absent from certain bacteria.

Professor J.S. Loutit (personal communication, 1971) has found that uptake of Tdr by Pseudomonas aeruginosa is very low and, in the work described here, very little Tdr was accumulated by two strains of P. fluorescens. Only 2 to 3%

and, in one experiment, 7% of the cells became lightly labelled - one grain per cell (Fig. 2.5; Table 2.8). This labelling could have been from background grains being coincidentally over bacteria. Alternatively the low level of uptake may have been due to incorporation of Tdr into compounds other than DNA.

The exact mechanism involved in these bacteria is not clear but the results suggest that the possibility of Tdr kinase being absent in certain bacteria should be taken into account when interpreting field data.

The effect of cell permeability on Tdr uptake (iii) above may also be important in some specific bacteria but again this is an unknown factor.

These three situations, (i), (ii) and (iii), may all reduce the level of incorporation of Tdr into DNA and thus the apparent number of cells about to divide. On the other hand, an over-estimate of the level of cell division may be formed if a detectable amount of Tdr is being incorporated into compounds other than DNA in cells which are not undergoing DNA replication.

In these experiments no biochemical extraction procedures were carried out before exposing slides to the emulsion, to ensure only those cells where Tdr was incorporated into DNA produced an autoradiogram.

Thymidine phosphate sugar compounds have been isolated from Lactobacillus acidophilus and E. coli (Okazaki, 1959), and Kornfeld and Glaser (1960) have shown that cell-free extracts of P. aeruginosa incubated with Tdr 5'- triphosphate and α-glucose 1-phosphate form Tdr diphosphate glucose which can be converted enzymically to Tdr diphosphate rhamnose.

Thus, any labelling of P. fluorescens could have been due to incorporation of Tdr into a sugar compound.

However, Professor Loutit notes that in P. aeruginosa very little Tdr gets into the cell at all and Fangman (1969) has shown that in a mutant E. coli strain which does not break down Tdr to thymine, Tdr uptake into DNA is very specific. The error introduced by not extracting the preparations is probably small compared to that from lack of uptake of Tdr.

Thus, there are a number of problems, whose significance is largely unknown, in using Tdr as an indicator of bacterial DNA replication in a mixed population. At present, interpretations of Tdr uptake data must be limited.

f) Type of emulsion and background grains

The emulsion used for the majority of the preliminary experiments was K2, which had a shelf life of 2 months after which the background grains built up to high levels. This emulsion has a relatively low sensitivity but is well suited to detect β radiation from tritium, which is of low maximum energy, 18 KeV, and rapidly loses energy on contact with AgCl.

L4 emulsion has a longer shelf life, at least 4 months, and build-up of background grains due to ageing of the emulsion is not such a problem. This emulsion, although it is less likely to form grains spontaneously, is more sensitive to radiation than the K2 emulsion. One result of this greater sensitivity is so-called tracking or formation of several grains from one β emission. This is a serious disadvantage if grain counts are required, but for the type of

counts made here it was not a problem. In fact, if it occurred, it enhanced the labelling over the cell.

The smaller grain size of this emulsion meant that more of a small cell was visible around any grains formed. However, the grains could still be resolved with the light microscope.

On the whole the advantages of L4 emulsion for autoradiography with the light microscope are considerable. The greater sensitivity, small size of grains and much lower background level of grains all made it a more suitable emulsion than K2 for the type of experiments undertaken - particularly the field experiments to be considered later. As L4 emulsion is more sensitive than K2, the incubation and exposure periods, and isotope concentrations determined with K2 emulsion will be satisfactory for L4, although these conditions may not be the minimal ones for detecting metabolizing cells with L4 emulsion.

Apart from the background grains which increase in numbers as the emulsion ages, background grains may also be caused by insufficient washing of the sample to remove excess radioactivity, and by reactions between the emulsion and chemicals, such as those used as fixatives.

Controls fixed with Lugol's iodine before labelling had no grains over the bacteria, except on slides where the background level was high, showing that this fixative does not react with the emulsion. However, 3% glutaraldehyde in phosphate buffer produced a high background level of grains if the sample was washed as a smear. If the sample was washed by centrifugation before being dried on to a slide,

this fixative was then removed satisfactorily and the background level of grains was low.

Provided these fixatives are removed before bacteria come in contact with the emulsion, both are satisfactory for autoradiographic experiments. Whether there was any loss of labelled material on fixation was not tested, but Hobbie and Wright (1965) mention that some loss may occur when Lugol's iodine is used for fixation.

Autoradiograms of unlabelled, live cultures of bacteria indicated that spontaneous labelling caused by reaction between the cells and the film, as reported by Waid, Preston and Harris (1971) for certain brewing yeasts, did not occur.

Excess radioactivity in the sample proved to be a serious problem in autoradiograms of field material. Thorough washing of the material before and after making a smear was essential if a low background was to be obtained.

g)Summary

Experiments with pure cultures of bacteria showed that optimal detection of labelling with ^{3}H-glucose and ^{3}H-Tdr could be obtained after 2 h incubation with an isotope concentration of 20 μCi/ml and exposure of the autoradiogram for 14 days. Washing the sample thoroughly after labelling was important. L4 emulsion appeared more suitable for autoradiograms at the light microscope level than K2 provided grain counts were not required. Interpretation of results from autoradiograms especially of field material could be difficult if an unknown proportion of the population could not take up the labelled compound.

Thus, the routine procedure used for preparing later

autoradiograms was as follows:

(i) Bacteria were labelled with 20 μCi/ml isotope.

(ii) After 1.75-2 h incubation, the sample was fixed with Lugol's iodine.

(iii) In most cases the sample was washed by centrifugation at this point.

(iv) A smear of the bacteria was prepared on a subbed slide.

(v) The smear was washed in running water.

(vi) The emulsion was melted and diluted 1:1 with distilled water.

(vii) The slides were dipped in emulsion and placed in a light-tight jar.

(viii) After drying for 2 h at room temperature, they were exposed at 4°C for up to 14 days.

(ix) The autoradiograms were developed, stained with 0.3% methylene blue, and in most cases mounted permanently with a coverslip (see p. 82 for mounting procedure).

Methods of preparing field material for autoradiography

a) Water samples

Ideally it is desirable to alter the sample as little as possible before labelling, but in the case of the lake water samples, the methods tested were all that were feasible. The bacteria of the lake water had to be concentrated to ensure there were sufficient bacteria in a microscope field to count. Several methods of preparing direct autoradiograms of membrane filters were tried but all were unreliable.

An alternative method was to concentrate the water by membrane filtration, after which the bacteria were scraped

from the filter on to a glass slide with a small piece of metal. Microscopic examination showed that very little material remained attached to the scraper. In several experiments differential filtration was carried out, to remove the algae. Large volumes (150 ml), which could not be labelled *in situ* economically, were filtered through first a 10 μm filter and then a 0.45 μm filter. The filters were cut up, to allow one sample to be treated in several ways, and transferred to bijou bottles containing isotope for labelling. A smear of bacteria and algae scraped from the labelled filters was then made.

Smaller samples could be labelled economically before concentrating. These labelled samples (4-6 ml) were centrifuged first at 650 g for 5 min to sediment out the algae. The supernatant was either filtered through a 0.45 μm filter (13 mm diameter), or concentrated by centrifuging in a 2.5 ml centrifuge tube, after which the sample was transferred to a slide for autoradiography. The sedimented algae were also concentrated further in a small volume centrifuge tube before a smear was prepared.

In one experiment when uptake of labelled compounds by bacteria attached to algae was being investigated, the algae were concentrated by centrifuging 10 ml of lake water for 5 min at 160 g before labelling. The supernatant was pipetted off leaving approximately 0.1 ml of water in which the algae were resuspended. The algae and lake water were added to an equal volume of isotope for labelling. The labelled sample was washed by three cycles of suspending in filter-sterilized water and centrifuging before a smear of the concentrated algae and bacteria was prepared.

For Lake Grasmere, where high numbers of algae often obscured bacteria, the best method of preparing a smear of the bacteria for autoradiography for future work would be to filter up to 150 ml of water through first a 10 μm and then a 0.45 μm filter, and after labelling to scrape the filters and then concentrate the material further in small volume centrifuge tubes. This would allow the samples to be well washed, with several cycles of centrifuging and re-suspending, which is essential if a high background level of grains is to be avoided.

Labelling samples before concentrating would be more representative of the state on sampling, but it was difficult to concentrate the bacteria sufficiently after the samples had been washed several times. This method would be satisfactory for lakes with a larger bacterial population.

b) Mud

Mud was labelled by adding a drop of mud to 0.5 ml (20 μCi/ml) of isotope. Part of the sample was then washed by mixing with filter-sterilized water. After letting the larger particles settle the supernatant was concentrated by centrifuging and a smear made. It is possible that many bacteria were attached to the larger particles which were allowed to settle. However, if the larger particles were included in the smear they often obscured the bacteria. The best way of obtaining a more representative smear would have been to shake the sample thoroughly or macerate it before decanting off the supernatant.

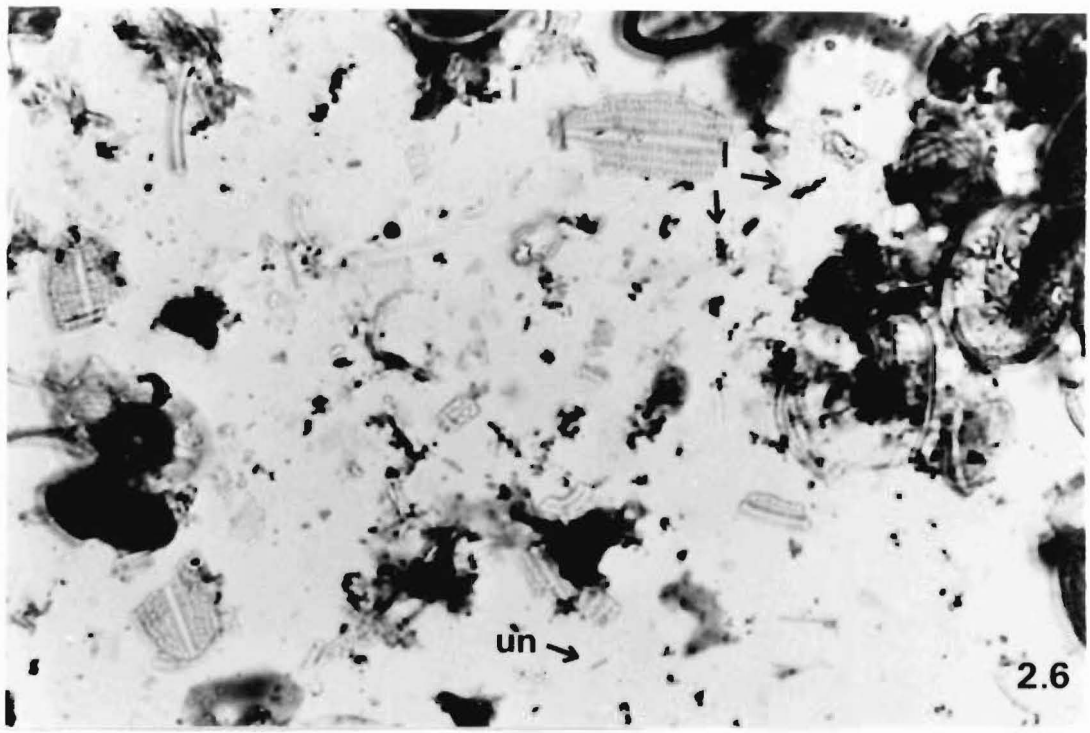
c) <u>Elodea canadensis</u>

Leaves were detached from the stem and labelled, in groups

of up to 10, in bijou bottles of isotope. The bacteria were scraped from 2 to 3 leaves on to a sterilized glass slide. The addition of several drops of filter-sterilized water helped remove the bacteria. The suspension was then transferred, with a Pasteur pipette, to a small volume centrifuge tube and was washed by one or two cycles of suspending in filter-sterilized water and centrifuging before a smear was made. This method produced very satisfactory autoradiograms (Figs. 2.6 and 2.7).

Autoradiograms of the smears were then prepared as described on p. 79, steps (v) to (ix). Both L4 and K2 emulsions were used in some experiments and L4 proved far more suitable. The low background of L4 was especially useful for field samples where the percentage of labelled cells was low.

After photographic processing, the smears were stained for 5 min in methylene blue. Five minutes staining rather than 30 s used for staining the autoradiograms of pure cultures of bacteria ensured that the algae in the field samples were well stained. When dry, the autoradiograms were permanently mounted using a modification of Bélanger's method (1961). Cedarwood oil was placed on the film for 45 min to clear the algae. The oil was drained off and 1:1 DePex and xylene applied for 20 min. The autoradiogram was then mounted under a coverslip in 3:1 DePex and xylene.



FIGS. 2.6 and 2.7 Autoradiograms of ^{3}H-glucose-labelled bacteria from moribund Elodea leaves.

Fig. 2.6 - labelled (l) and unlabelled (un) cells are visible; x1 250 Fig. 2.7 - fixed with lugol's iodine before labelling; only unlabelled cells are visible; x 1 250

Method of preparing autoradiograms for the electron microscope

Autoradiograms of field material can be difficult to interpret if many small bacterial-sized particles are present in the sample. As a check of the counts of labelled cells in autoradiograms for the light microscope (LM), autoradiograms of a few selected samples were prepared for high resolution autoradiography. With the higher magnifications, bacteria can be more easily distinguished from other small particles. Successful EM autoradiograms were obtained using the following methods.

a) Incubation and fixation

Samples were incubated for 1.75-2 h with 20 μCi/ml of isotope. They were then fixed with 3% glutaraldehyde in phosphate buffer pH 7. This fixative did not cause additional grains in LM autoradiograms if samples were well washed (p. 77). It was better than Lugol's iodine for EM work, because fixation with iodine made the cells more electron dense and this might reduce the clarity of grains formed over the bacteria.

b) Staining and preparation of grids

EM autoradiograms of pseudomonad 6W 5 and Elodea bacteria were prepared. Pseudomonad 6W 5 was stained after concentrating by centrifugation. Bacteria were scraped from the leaf samples as described on p. 82, and washed with two cycles of centrifuging and resuspending before staining. They were negatively-stained with PTA and applied to the grids as described on pp. 34-35, except that in some cases staining was extended to 3-4 h.

Grids of bacteria were set up for autoradiography after

some preliminary preparations had shown that the concentration of bacteria was satisfactory. These preliminary grids, which had been exposed to the electron beam, were not used for autoradiography.

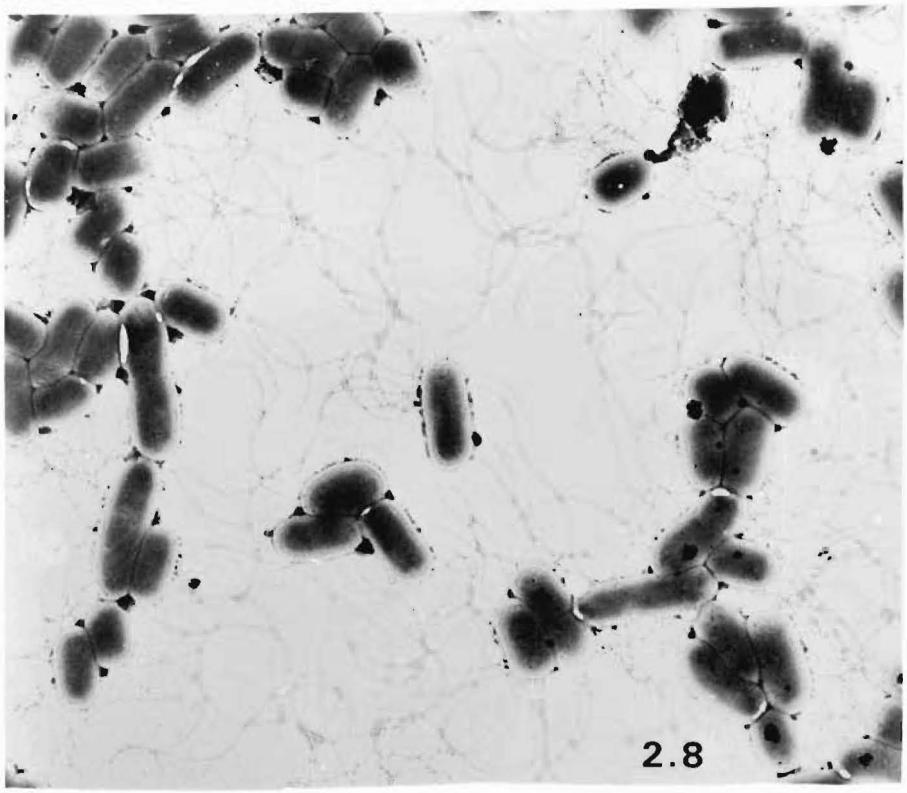
Preparations of negatively-stained bacteria with and without an emulsion layer over them were examined to determine the extent of loss of resolution when emulsion was placed over unsectioned bacteria. While detail such as flagella could not be resolved when emulsion was present, well stained bacteria were still clearly distinguishable so it was decided sectioning was not necessary (Figs. 2.8 and 2.9).

c) Application of emulsion to grids

The looping method of Caro and van Tubergen (1962) was used with some modifications. Ilford L4 emulsion, melted (see p. 65), was diluted with three times its volume of distilled water. 2.7 ml of diluted emulsion was sufficient for eight grids.

The diluted emulsion was poured into a Petri dish which was stood in a Petri dish lid. Hot water was run into the lid to keep the emulsion at about 40°C. With a platinum/palladium loop, 4.5 cm in diameter, a film was picked up and allowed to dry with continual rotation of the loop. Moving the loop ensured that the film did not burst before it had nearly gelled. Although this introduced uneven swirls in the film, a uniform area could be found to place over the grid.

The loop, with the film of emulsion just beginning to gel, was touched down on to a grid, which was on a brass stub 3 mm in diameter. Using this procedure a film, which when examined with the EM without photographic processing could



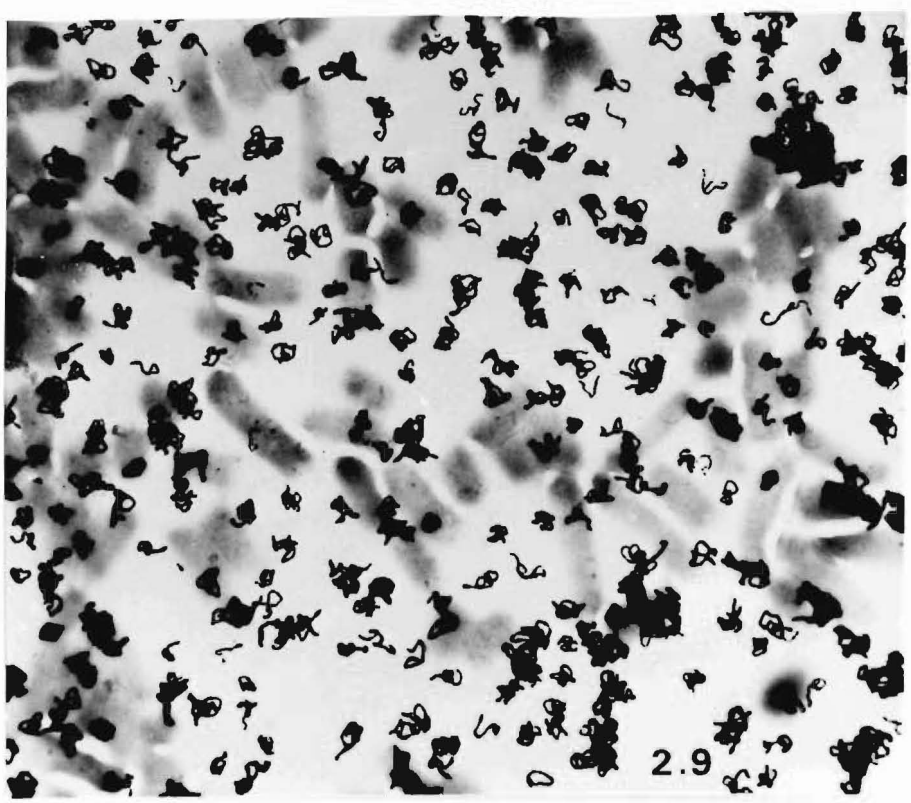


FIG. 2.8 Bacteria, negatively-stained with PTA, with no emulsion over them; x 9 500.

FIG. 2.9 Bacteria, negatively-stained with PTA, with emulsion placed over them when nearly gelled; exposed to light briefly and developed. Bacteria are clearly distinguishable and silver grains can be seen on top of some of the bacteria; x 9 700.

be seen to be near to a monolayer, was produced over the bacteria. Fig. 2.9 shows a grid which was exposed to light briefly before developing and grains can be seen on top of some of the bacteria. If the emulsion was applied in a more liquid state, the emulsion did not remain over the bacteria (Fig. 2.10). After applying emulsion to a grid, the loop was wiped to remove pieces of dried emulsion from the previous film.

The emulsion coated grids were attached by the edge with tape to glass slides. The slides of grids were placed in an anaerobic jar and dried, as described on p.79 part (viii), before exposing at $4^{o}C$. Because of the longer exposure periods involved, they were exposed in an atmosphere of nitrogen to prevent any latent image fading.

d) Exposure period and treatments used

The emulsion layers used in EM autoradiograms are less sensitive to β radiation, because they are so thin, and far longer exposure periods are required than those used for LM autoradiography. Caro and van Tubergen (1962) suggested that EM autoradiograms should be exposed for 10 times as long as LM autoradiograms of the same material.

To avoid long exposure periods of up to six months, a sample of ^{3}H-glucose-labelled cells of pseudomonad 6W 5, which had produced a good autoradiogram for the LM after a 3 day exposure period (Table 2.8), was used to prepare EM autoradiograms. Elodea leaf samples labelled with ^{3}H-Tdr and ^{3}H-glucose were also examined.

The pseudomonad was exposed for 4 and 8 weeks and the ^{3}H-Tdr-labelled Elodea bacteria for 4, 8 and 12 weeks, with

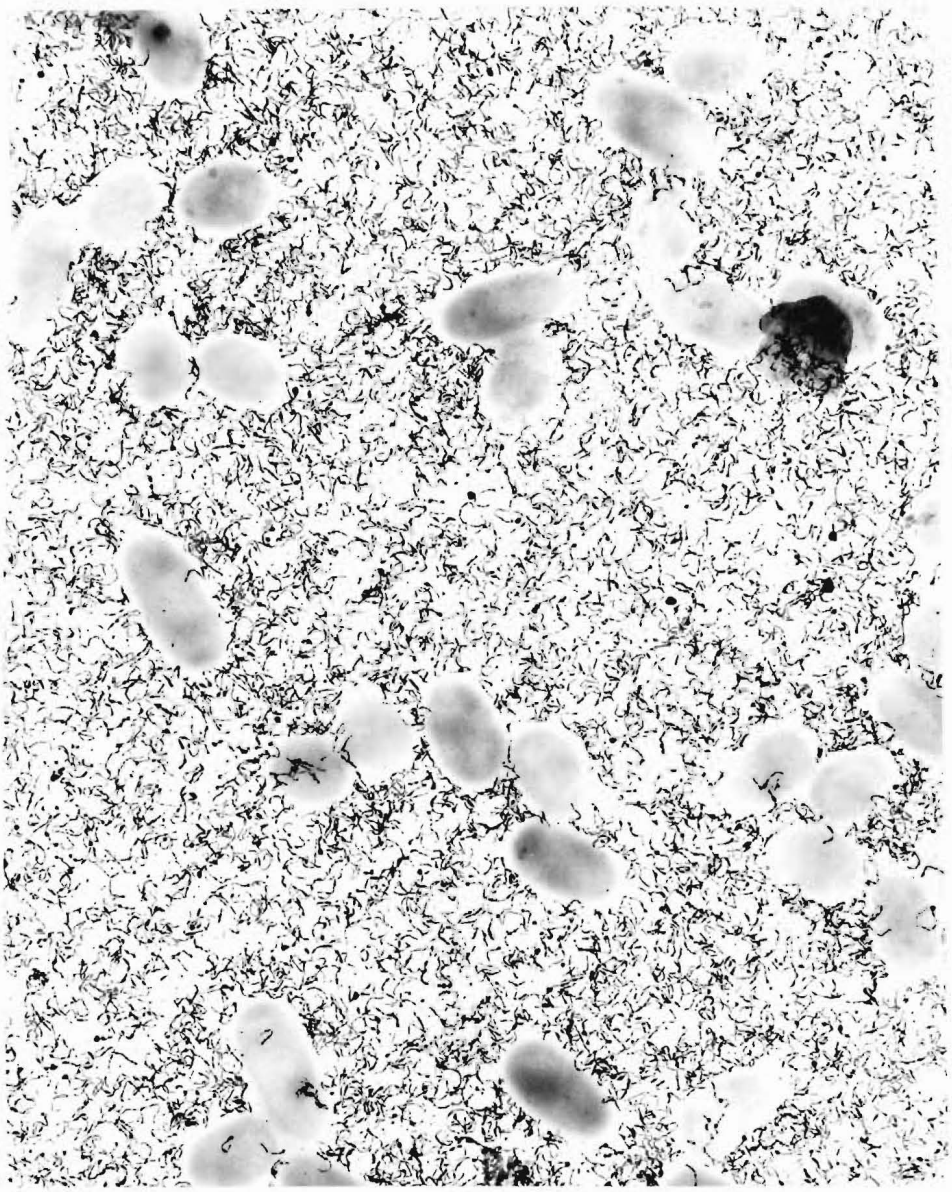


FIG. 2.10 Bacteria, negatively-stained with PTA, with emulsion placed over them while liquid; exposed to light and developed.

Few grains can be seen over bacteria; x 17 000.

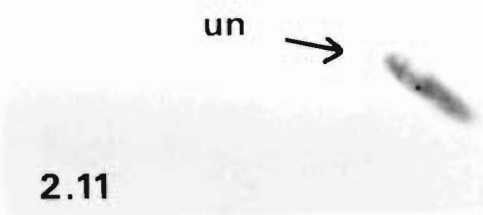
3 grids for each treatment. Two grids of ^{3}H-glucose-labelled leaf bacteria were exposed for 12 weeks only. Controls of leaf bacteria fixed before labelling with ^{3}H-Tdr and ^{3}H-glucose were set up in triplicate and examined after 12 weeks. A blank grid was included for each exposure period to check the level of background grains.

e) Photographic processing and examination of grids

The slides, with grids attached, were processed as described by Caro and van Tubergen (1962) viz. Microdol - 5 min; 1% acetic acid - 10 s; May and Baker Super Amfix diluted 1:6 - 5 min; tap water wash - 6 min, 4 changes. Surplus water was removed with filter paper and as soon as the grids were dry they were transferred to a grid box. If the grids were left on the slides for any length of time they became very difficult to remove. Before examining, the grids were dried under vacuum over phosphorus pentoxide. The grids were then examined and the numbers of labelled and unlabelled cells were counted. Figs. 2.11 and 2.12 show examples of labelled and unlabelled bacteria and the low background level of grains even after exposure for 12 weeks. No labelled bacteria were seen on the control grids. The sample size was determined as described on p. 66. One compostite count from the replicate grids was made. Grids could only be examined once or twice before the film collapsed.

f) Comparison of counts of labelled cells in LM and EM autoradiograms

The counts of labelled cells in LM and EM autoradiograms are shown in Table 2.9. These were only strictly comparable



2.11

2.12

FIGS. 2.11 and 2.12 EM autoradiograms of bacteria from moribund Elodea leaves.

Fig. 2.11 - labelled with ^{3}H-thymidine and exposed for 8 weeks, showing labelled (l) and unlabelled (un) cells; x 16 000. Fig. 2.12 - labelled with ^{3}H-glucose and exposed for 12 weeks, showing different degrees of labelling; x 20 500.

for pseudomonad 6W 5, when the same bottle of labelled cells was used to prepare all the autoradiograms. For the leaf bacteria, each batch represents a sample of 2 to 3 leaves, all of which were moribund leaves from the same stem. Thus, the EM and LM autoradiograms were prepared from different batches of leaves. However, the EM autoradiograms of ^{3}H-Tdr-labelled bacteria which were exposed for different lengths of time originated from one batch of leaves. Three counts on different areas of each of the LM autoradiograms were made and the mean percentages of labelled cells with their standard deviations are shown in Table 2.9.

Two points were being tested in these experiments. These were the length of the exposure period required for the EM autoradiograms to detect all metabolizing cells, and the validity of the counts of labelled cells in the LM autoradiograms. The counts of labelled cells in the EM autoradiograms of pseudomonad 6W 5 exposed for 4 and 8 weeks were compared with a chi-square test (Snedecor and Cochran, 1967). There was no significant increase in counts after the longer exposure period. The natural variability in the distribution of labelled cells within the batch was checked from three counts from different areas of the LM autoradiogram. These data were analyzed using the variance test for homogeneity of the binomial distribution (Snedecor and Cochran, 1967) and no significant difference was detected. The counts from the EM autoradiograms exposed for 8 weeks were then compared with all the LM autoradiogram counts and they were found to be significantly different at the 1% level. This result suggested that the counts from the LM

TABLE 2.9 Percentage of labelled cells in LM and EM autoradiograms

Sample	Isotope[1]	Batch	% bacteria labelled exposure period (weeks)				
			LM			EM	
			1	1.86	4	8	12
pseudomonad 6W 5	Glu	I	92±1		80	83	
		I					75
		II		73±4			
Elodea leaf bacteria	Glu	III		83±2			
		IV		90±1			
		V dead control					0
		I			65	70	72
		II		60±2			
Elodea leaf bacteria	Tdr	III		69±0.5			
		IV		72±2			
		V		74±2			
		VI dead control					0

[1] Glu - ^{3}H-glucose; Tdr - ^{3}H-thymidine.

autoradiogram might be underestimating unlabelled cells. As this particular sample had a high proportion of labelled cells, it is possible that the labelled cells may have been masking some unlabelled ones. It is therefore important that there should not be clumps of bacteria in the preparations.

The chi-square test was applied to counts from the three exposure periods of the ^{3}H-Tdr-labelled bacteria from Elodea leaves. The increases in counts of labelled cells after 8 and 12 weeks exposure were not significant. Unfortunately no similar comparison was available for the Elodea bacteria labelled with ^{3}H-glucose. Further data are required before any definite conclusion can be drawn on the exposure period needed to ensure all bacteria, which have accumulated some label, have formed an autoradiogram. However, these experiments suggested that the majority of bacteria from Elodea which took up ^{3}H-Tdr had formed an autoradiogram after 4 weeks, but that to detect all metabolizing bacteria a longer exposure period might be required.

The EM autoradiogram counts of ^{3}H-glucose- and ^{3}H-Tdr-labelled bacteria from Elodea were not strictly comparable with the LM counts. Counts of LM autoradiograms prepared from several batches of leaves suggested that there was greater variability between batches than in one sample (Table 2.9). An analysis of variance of the ^{3}H-Tdr data confirmed this. The distribution of labelled cells within each sample appeared, however, to be very uniform as shown by the small standard deviations of the counts from each slide.

These results indicated that to compare the counts of labelled cells in EM and LM autoradiograms of leaf bacteria a single batch of leaves would have to be used for all the autoradiograms. However, both the ^{3}H-glucose and ^{3}H-Tdr counts from the EM autoradiograms were within the range of counts obtained from the LM autoradiograms of different batches of leaves and it seems likely that, provided bacteria are not clumped, valid counts of labelled bacteria can be obtained from LM autoradiograms.

High resolution autoradiography can thus be used to detect the proportions of bacteria which were likely to have been metabolizing in situ. This method has advantages over LM autoradiography when bacteria are lightly labelled as grains are more clearly visible. However, preparation of material takes longer and the autoradiograms cannot be examined for several months. Higher concentrations of isotope might enable a shorter exposure period to be used.

g) Summary

(i) Uptake of labelled compounds by a pure culture of a pseudomonad and a mixed bacterial population from moribund Elodea leaves was detected successfully by high resolution autoradiography. Bacteria, which were not sectioned, were negatively-stained and applied to EM grids. L4 emulsion was then placed over the bacteria using the looping method. Grids were exposed for 4-12 weeks.

(ii) Counts of labelled cells in LM and EM autoradiograms of similar material suggested that valid counts of labelled cells in LM autoradiograms could be obtained provided bacteria were not clumped.

CHAPTER 3

THE BACTERIAL POPULATION OF LAKE GRASMERE

This chapter describes the seasonal variation in numbers and kinds of bacteria in the water from three areas of Lake Grasmere and considers some of the factors which could be influencing these bacteria. The three areas of the lake studied were open water, water over *Elodea canadensis* and water over the harbour spring. These were chosen because such factors as inflow of water, weed beds and plankton blooms could be influencing their bacterial populations to varying extents. By comparing the bacterial populations in the different parts of the lake, the effect of these factors on the bacteria could be studied more closely.

3.1 METHODS

A total of 51 samples were collected on 21 occasions between April, 1969, and December, 1971. The numbers of bacteria in all samples were estimated by the pour plate method. All samples were plated within 2 h of sampling except for the open water sampled in April, 1969, which was plated within 7 h of sampling. A range of bacteria from 15 samples was characterized. Bacteria were characterized from all three areas of the lake in March, 1970. On six other dates bacteria from two of the three areas only were characterized (Appendix 1). More bacteria were characterized from

the samples collected in April and June, 1969, than the other samples, because different media were being tested and the results from all the media were pooled.

In four samples, bacteria were also counted directly. These samples were macerated and filtered as described on p. 53. The sample collected on 5 December, 1971, was scraped from the filter on to a glass slide before counting.

Details of sampling dates, areas sampled and methods used are given in Appendix 1.

3.2 RESULTS

Size and fluctuations of the bacterial population in different parts of Lake Grasmere

The numbers of viable bacteria in samples collected between April, 1969, and February, 1971, are shown in Fig. 3.1. Although the maximum number of bacteria per ml reached between 1090±70 and 4360±850 in the different areas of the lake, populations between 100 and 500 bacteria per ml were found in 31 of the 51 samples and the order of magnitude of the population in the three areas was similar.

At certain times of the year, there were peaks and troughs in the bacterial population at the same time in all three areas of the lake, for instance, from September, 1970, to February, 1971. At other times, for example, between March and August, 1970, fluctuations in the bacterial populations in the different areas appeared to be independent of each other.

Estimates were made of the bacterial population of four open water samples by indirect and direct methods (Table 3.1). The direct counts provided a figure for the total

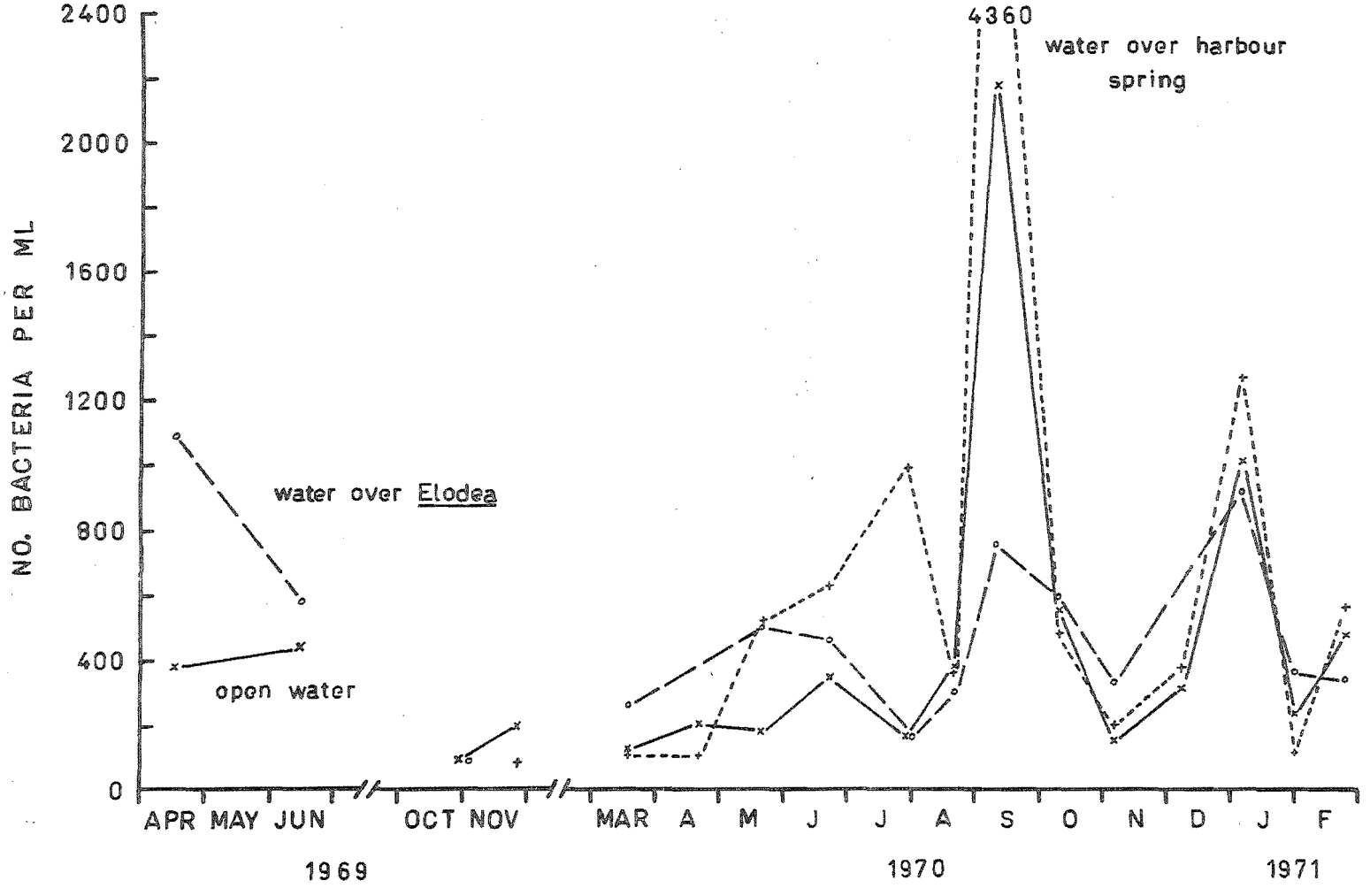


FIG. 3.1 Seasonal fluctuations in numbers of bacteria in lake water

TABLE 3.1 Comparison of indirect and direct bacterial counts

Date sampled	No. bacteria (per ml) plate count	direct count	Plate count as proportion of direct count (%)
7 April	242± 92	1020± 246	24
19 April	228± 40	903± 296	25
13 July	218± 93	651± 245	34
5 December	540±125	2680±1340	20

bacterial population in the water but because of the difficulties in counting the bacteria, described on p. 53, this was probably a minimum value. Bacteria were more easily counted when they had been scraped from the filter on to a glass slide. However, the loss of bacteria in transferring the sample to the square and any uneven distribution of bacteria on the slide, both meant that even this count was still a rough estimate only. The results showed that the plate counts were only estimating a small part of the bacterial population.

Kinds of bacteria in different parts of Lake Grasmere

Pseudomonads were found in all the samples and frequently made up more than 20% of the bacteria characterized (Table 3.2). Other kinds of bacteria which were found in

TABLE 3.2 Kinds of bacteria in the lake water

Date sampled	Sample	No. characterized	Percentage distribution											
			Alcaligenes/ Achromobacter	pseudomonads	flavobacteria	Cytophaga	Vibrio extorquens	Enterobacteriaceae	Aeromonas/ Vibrio	Acinetobacter	Chromobacterium	coryneforms	Micrococcaceae	Bacillus
15/ 4/69	Open water	96	6	8	33	12	27			+		7	+	+
15/ 4/69	Over Elodea	67	12	25	18	21	6	+				8	6	
17/ 6/69	Open water	74	+	15	13	22	8	9	+	+		12	10	+
17/ 6/69	Over Elodea	72	+	8	21	8	24	+				22	13	
28/10/69	Open water	23		43	+							13	22	17
28/10/69	Over Elodea	46	+	61	+							29	+	
25/11/69	Open water	45	7	33	18	7		+	+			25	+	+
25/11/69	Harbour[1]	43	+	31	7	34		7	+			10	+	
17/ 3/70	Open water	51	+	23	+			51	20					
17/ 3/70	Over Elodea	47	11	32	+			34	11	+		+	+	+
17/ 3/70	Harbour	49	17	36	+			25	14	+		+		+
20/ 4/70	Open water	48	31	21				33	15					
20/ 4/70	Harbour	49	14	59	+			10	10			+		
13/ 7/71	Open water	44	+	28	22		8		+		+	33		+
13/ 7/71	Over Elodea	41	+	11	25						+	56	+	

+ $\leq 5\%$

[1] Harbour - water from over the harbour spring

most samples were Alcaligenes/Achromobacter, flavobacteria and coryneforms. The kinds of bacteria in different areas of the lake sampled at one time were often basically similar but there were usually some differences, such as the number of bacteria in certain groups or the absence of one group. Differences were also found in the kinds of bacteria in the lake at different times of the year. For example, the samples taken in November, 1969, had more flavobacteria, Cytophaga and coryneforms, but fewer Aeromonas/Vibrio and Enterobacteriaceae than the samples collected in March and April, 1970.

3.3 DISCUSSION

Size of the bacterial population in different parts of Lake Grasmere

The bacterial population of Lake Grasmere, as estimated by the plate method, was not large. It is hard to generalize on the size of a population which often fluctuates widely but typical numbers of bacteria in other lakes, as estimated by the plate method, are around 10 000 bacteria per ml for the eutrophic Ekoln basin of Lake Mälaren (Fondén, 1969a) to from an average of 148 bacteria per ml in open water to an average of 1023 bacteria per ml in a shallow bay of the large oligotrophic Flathead Lake (Graham and Young, 1934). However, Potter and Baker (1961) reported even lower numbers of bacteria in Flathead Lake. They found from 3-66 bacteria per ml at different depths of the open water when they sampled

in 1953 and 1954. Comparing these figures with the data from Lake Grasmere, the bacterial population of Lake Grasmere is on average a little larger than that of Flathead Lake.

The direct counts of bacteria in lake water were only partly successful but they did serve to show that the total bacterial population of the water was at least three to five times higher than the plate count estimate. The ratio between direct and indirect counts of bacteria in a 'rather clean' section of a river obtained by Jannasch (1958) was of the same order of magnitude.

Fluctuations in the bacterial population in different parts of Lake Grasmere

The similarity in the order of magnitude of the bacterial populations from the three areas suggested that the concentration of available nutrients for bacteria was often the same. However, as fluctuations in the bacterial populations occurred independently at times it was apparent that the lake was far from homogeneous.

Analyses of the seasonal fluctuations in bacterial populations are undertaken in Chapters 4, 6, 7 and 9, but some of the possible causes of these fluctuations will be considered. An increase in the numbers of bacteria in all areas of the lake at the same time, as found in September, 1970, could be the result of a very heavy rainfall with consequent inflow of nutrients and bacteria to the lake. Alternatively, the amount of substrate available to bacteria in the lake could have increased with the dying off of a plankton bloom.

Fluctuations in the bacterial populations occurred independently in the various parts of the lake between April

and August, 1970. Such independent fluctuations are harder to analyze as usually several factors are interacting. For example, the bacterial population near the inlet might be responding to an inflow of nutrients but further out into the lake the bacteria might be influenced more strongly by the presence of weed or plankton.

Kinds of bacteria in different parts of Lake Grasmere

The kinds of bacteria found in the water samples appeared to vary more from one sampling date to another, than between samples from different areas of the lake collected at one time. However, in all the water samples, Gram-negative rods were predominant as previously noted by many authors (Taylor, 1942; McCoy and Sarles, 1969 and others). The differences in kinds of bacteria between the samples will be discussed in later chapters, when the possible factors causing these variations are analyzed.

This chapter has described the seasonal fluctuations observed in the bacterial population of the water of Lake Grasmere in general terms, so that these changes can be brought into perspective before any detailed analysis is made. The size of the bacterial population suggested that Lake Grasmere was a nutrient-poor lake. The majority of the bacteria were Gram-negative rods, typical of an aquatic habitat.

In the following chapters the fluctuations in bacterial populations will be examined in relation to certain parameters - rainfall, Elodea growth and plankton populations - all of which provide nutrients, directly or indirectly for the lake

bacteria. These factors were chosen as they are important sources of organic matter in Lake Grasmere. Other sources, such as pollen, and the possible importance of animals other than zooplankton, and fungi, as a link between primary production and bacterial decomposition, were not considered in this study.

3.4 SUMMARY

1) Numbers of viable bacteria in samples of lake water were of the same order of magnitude during the two and a half year period of study. The mean number of bacteria per ml in different areas reached from 1090 to 4360, but numbers were between 100 and 500 bacteria per ml in 31 of the 51 samples analyzed.

2) Direct counts of bacteria in lake water were carried out on four samples but were only partly successful because of the small number of bacteria present compared to the amount of detritus and numbers of algae. Counts 3 to 5 times higher than the plate counts of the same samples were obtained.

3) Pseudomonads were found in all the samples of lake water and often made up more than 20% of the bacteria characterized. Flavobacteria, coryneforms and Alcaligenes/Achromobacter were also common. Cytophaga, Vibrio extorquens, Aeromonas/Vibrio, Enterobacteriaceae and Micrococcaceae were numerous in some of the samples.

4) There was often a greater similarity in kinds of bacteria between samples collected at one time from different

areas, than between samples from one area taken on different dates.

CHAPTER 4

THE RELATIONSHIP BETWEEN RAINFALL AND BACTERIA IN LAKE GRASMERE

Heterotrophic bacteria in lake water are influenced by the amount of dissolved inorganic and organic nutrients, particulate matter, water and numbers of bacteria entering the lake. Rainfall in the catchment area of a lake can affect the quantity of all these parameters in inlets and, thus, the relationship between changes in bacterial populations in lake water and rainfall is often complex. An understanding of the influence of rainfall on the bacterial population of a particular lake is essential if fluctuations in bacterial populations are being studied.

Increases in the numbers of bacteria in a lake after rainfall have been recorded (Fred, Wilson and Davenport, 1924; Graham and Young, 1934; Taylor, 1940, 1949; Collins, 1960, 1963; Collins and Willoughby, 1962; Chen, 1968; Fondén, 1969a; Woodbridge and Garrett, 1969; Jones, 1971). These increases have been attributed to a combination of inflow of bacteria and multiplication of bacteria in the lake, due to the increased nutrients available (Collins, 1960). When there are sudden large increases in the numbers of bacteria following heavy rain, washing-in of bacteria is thought to be the major factor responsible. For instance, Collins and Willoughby (1962) concluded that the entry of particulate matter with its attached bacterial flora was

responsible for an increase in bacterial numbers from below 3 000 to between 23 000 and 40 000 bacteria per ml. This increase occurred within two days of a rainfall of 8.25 cm: three days later the bacterial population had dropped to between 10 000 and 18 000 bacteria per ml and, after another seven days, populations between 2 000 and 9 000 bacteria per ml were recorded. The rapid decrease was attributed to a loss of particulate matter through the outlet stream. Evidence that the majority of bacteria in inflowing waters was attached to some solid substrate was provided by Jannasch (1956), who found that very few bacteria were not attached. For example, in a sample of water from the River Nile, only 0.04% of the total population was freely suspended.

After very heavy rainfall increases in the numbers of bacteria have been found throughout a lake, but often increases are confined to the areas of a lake near inlets (Taylor, 1940). The lack of an increase in the bacterial population further out into a lake has been ascribed to a combination of sedimentation of particulate matter and attached bacteria, dilution of bacteria by the main body of the lake and death because of unfavourable conditions (Taylor, 1940).

The nutrient status of a lake itself will also influence the effect of any inflow of water on lake bacteria. In a study of the eutrophic Ekoln basin of Lake Mälaren in Sweden, Fondén (1969a) found that the level of production of organic matter and, consequently bacterial decomposition, in the lake determined whether inflowing bacteria dominated the lake microflora. In winter when there was little production of organic matter in the lake, a reduced inflow of

bacteria into the lake resulted in a reduction in the numbers of bacteria in the lake. However in summer, production of organic matter within the lake was the dominating factor and the numbers of inflowing bacteria bore little relation to the fluctuations in the bacterial population of the lake. This study did not take into account the effect of inflowing nutrients on bacterial multiplication in the lake. Taylor (1949) considered that increases in numbers of bacteria after rainfall were primarily due to the promotion of growth rather than washing-in of bacteria and there can be little doubt that any long term increases in bacterial numbers following rainfall must be due to the addition of nutrients. The effect of an inflow of nutrients on the lake bacteria will also be influenced by the nutrient status of the lake. Jones (1971) concluded that a positive correlation between bacterial numbers and rainfall found in a nutrient-poor lake was due to a limitation of nutrients in the lake which was alleviated by rainfall. However, in a nutrient-rich lake the correlation between bacterial numbers and rainfall was not significant. A lower concentration of nutrients in the water flowing into a river may result in the bacterial population of the river being reduced due to a dilution effect (Taylor, 1941).

Thus, the effect of inflowing nutrients and bacteria on the bacteria in a lake will vary according to the particular characteristics of the lake. In addition, within one lake the relationship between amount of rainfall and numbers of bacteria will not be constant all the year round. Fred, Wilson and Davenport (1924) noted that the rate of runoff of water was influenced by the state of the ground. For

instance, when the ground was frozen, there was a rapid runoff and reduced leaching of nutrients. The temperature of the inflowing water also affected the response of lake bacteria, with the greatest increase in numbers being found when the temperature of the inflowing water was nearest to the lake temperature.

As with all ecological factors, rainfall cannot be considered as a single factor. The interaction of rainfall with other factors of the environment, such as temperature and particulate content of the inflow, nutrient level of the soil, and also biological factors in the lake itself, such as weed growth and plankton blooms, must also be considered when studying fluctuations in populations of lake bacteria.

In this chapter the numbers and kinds of bacteria in the main body of the lake and the inlets are considered and the relationships between these bacterial populations and rainfall and nutrient levels are discussed.

4.1 METHODS

Daily rainfall was measured by an automatic rain gauge at a station about 2 km to the west of the lake and the results were recorded by the North Canterbury Catchment Board. The total rainfall in the 7 and 14 days before sampling was calculated.

Water samples collected between October, 1969 and March, 1971, are discussed in this chapter. Details of sampling dates and areas sampled are given in Appendix 2. The numbers of bacteria in all samples were estimated by the pour plate method and bacteria isolated from selected samples

were characterized. No direct counts of bacteria were made for these samples.

Uptake of radioactive glucose and thymidine by water bacteria was studied on two occasions. An open water sample was filtered through first a 10 μm filter and then a 0.45 μm filter, as described on p. 80, before labelling. A sample from inlet C was untreated before labelling. The samples were labelled and autoradiograms prepared as described on p.79, steps (i) to (ix). Step (iii) was omitted with the open water sample which was labelled attached to the filter.

Data on total organic nitrogen (Kjeldahl), nitrate nitrogen and soluble phosphate in the inlets and surface samples of open water were obtained from Dr V.M. Stout.

Correlation coefficients were calculated between the following data, after bacterial counts had been transformed as described on p. 21:-

a) rainfall in 7 days before sampling with the numbers of bacteria in

(i) the water over the harbour spring between November, 1969 and February, 1971,

(ii) the open water between November, 1969, and March, 1971, and

(iii) water over the weed between March, 1970 and February, 1971;

b) total organic nitrogen in water from over the harbour spring with the numbers of bacteria in water over the harbour spring between November, 1969, and February, 1971, excluding the bacterial sample collected on 20 August when chemical determinations were not made;

c) nitrate nitrogen in water over the harbour spring

with the numbers of bacteria in water over the harbour spring for the same samples as b);

d) total organic nitrogen in the open water with numbers of bacteria in the open water between November, 1969, and March, 1971, excluding the bacteria collected on 20 August.

4.2 RESULTS

Numbers of bacteria in the water over the harbour spring and in the main body of the lake related to

a) rainfall

The spring flowing into the harbour was the inlet most frequently sampled. Therefore, data from this inlet are compared with the data from the main body of the lake first and then the more limited data from the other inlets are considered.

The rainfall in the 7 days before sampling was found to be more closely related to the numbers of bacteria in the water over the harbour spring, than the rainfall in the 14 days before sampling. The rainfall for 7 days before sampling was therefore plotted with the numbers of bacteria in the open water, water over the weed and water over the harbour spring (Fig. 4.1).

There was a highly significant correlation between rainfall and the numbers of bacteria over the harbour spring, but between rainfall and numbers of bacteria in the open water and water over the weed, correlation coefficients were not significant (Table 4.1).

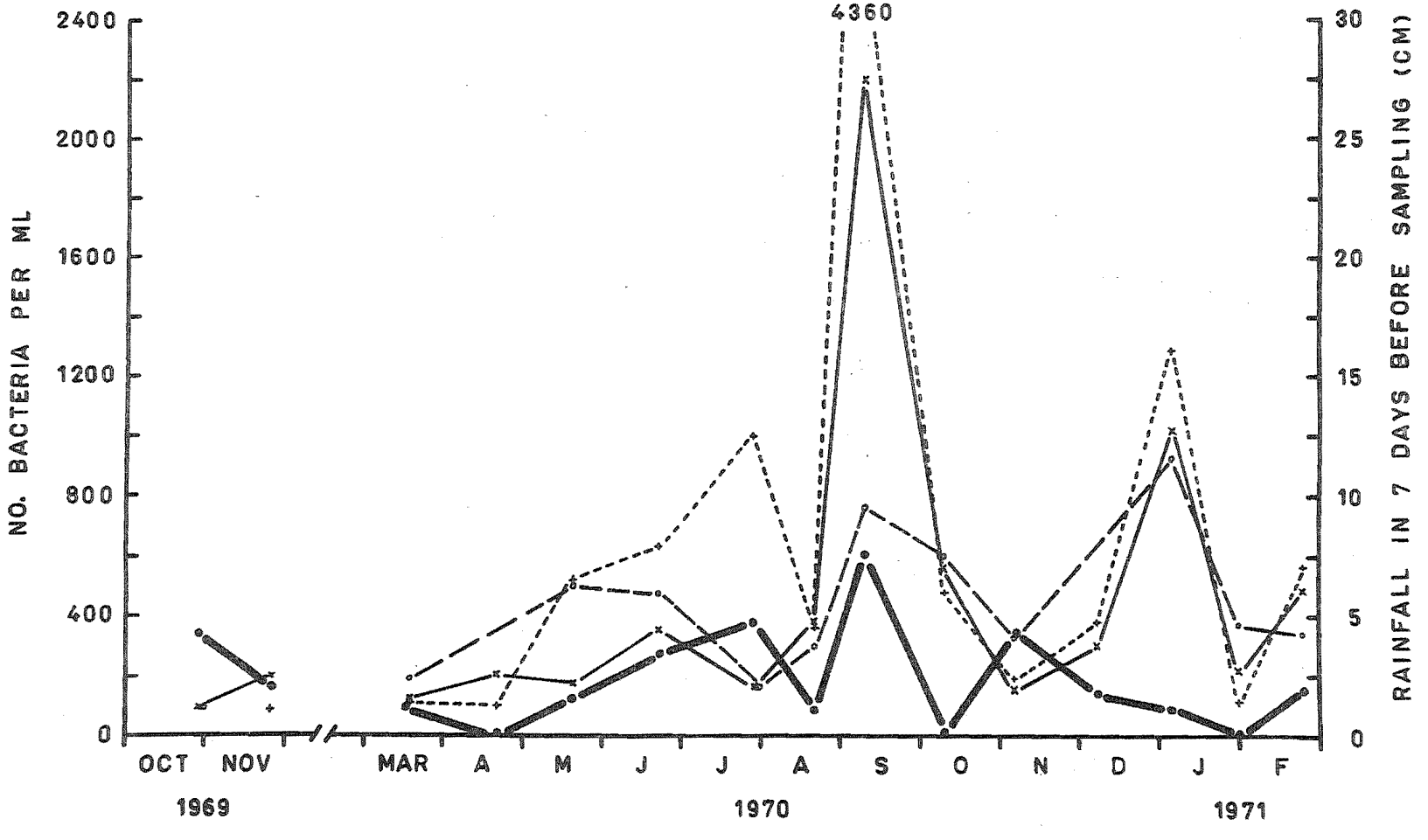


FIG. 4.1 Seasonal variation in numbers of bacteria in lake water and rainfall. Rainfall —●—; bacteria in open water —×— , water over Elodea ——○——, water over harbour spring----+----.

TABLE 4.1 Correlations between numbers of bacteria and physico-chemical factors

x No. bacteria in	y	No. pairs	Correlation sign and significance level
Water over harbour spring	Rainfall in 7 days before sampling	14	+ 0.01
Water over harbour spring	Total organic nitrogen in water over harbour spring	13	+ 0.05
Water over harbour spring	Nitrate nitrogen in water over harbour spring	13	ns[1]
Open water	Rainfall in 7 days before sampling	16	ns
Open water	Total organic nitrogen in open water	15	+ 0.01
Water over weed	Rainfall in 7 days before sampling	11	ns

[1] ns - not significant.

In Fig. 4.1 it can be seen that the fluctuations in numbers of bacteria in the water over the harbour spring followed closely the changes in rainfall between March and October, 1970, but during the summer the fluctuations in the bacterial population appeared to be independent of the amount of rainfall. Although rainfall and numbers of bacteria in the main body of the lake, as represented by the samples of water over the weed and open water, were not significantly correlated during the period of a study, a marked

increase in bacterial numbers after rainfall was recorded in September, 1970. In the 7 days before sampling, 7.56 cm of rain fell. The total rainfall for the 14 days before sampling was 28.4 cm. This continual heavy rain was reflected in the increase in numbers of bacteria throughout the lake (Fig. 4.1). At other times of the year, the fluctuations in bacterial populations in the main body of the lake appeared to bear little relationship to the amount of rainfall immediately before sampling.

There appeared, however, to be a relationship between the numbers of bacteria in the open water and the rainfall in the week before sampling combined with the rainfall over previous months (Table 4.2). Rainfall from August of the year before sampling in each case is given. These data showed that in 1968/1969, high winter rainfall and moderate summer rainfall combined with moderate rainfall before sampling led to relatively high numbers of bacteria in the open water in 1969. From August to November, 1969, rainfall was very low. Although it was higher between December, 1969, and February, 1970, there was little rain before sampling from March to June, 1970, and generally lower numbers of bacteria were found during this period. Rain in the latter part of 1970 was high and although little rain was recorded between December, 1970, and March, 1971, and before sampling in 1971, numbers of bacteria were not at such a low level as in 1970. Thus, the amount of rainfall in the previous winter months appeared to be a major factor controlling the size of the bacterial population in the open water in the following autumn.

TABLE 4.2 Relationship between rainfall and numbers of bacteria in the open water

Year	Rainfall (cm)		March		April		May		June	
	August-November	December-February	Rainfall[1] (cm)	No.bacteria (per ml)	Rainfall[1] (cm)	No.bacteria (per ml)	Rainfall[1] (cm)	No.bacteria (per ml)	Rainfall[1] (cm)	No.bacteria (per ml)
1968/1969	71	23		-	4.2	380 ± 120		-	4.6	440 ± 120
1969/1970	29	31	1.2	124 ± 16	0.9	207 ± 36	1.6	179 ± 38	3.4	350 ± 130
1970/1971	64	13	0.0	287 ± 84	0.8	240 ± 90		-	0.0	420 ± 64

[1] Rainfall in 7 days before sampling.

b) nutrient concentration

The fluctuations in concentrations of total organic nitrogen, nitrate nitrogen and soluble phosphate in the open water and water over the harbour spring between March, 1970 and February, 1971, are shown in Fig. 4.2. Phosphate concentrations were usually very low in both areas. The highest concentration was 0.016 mg P/l, which was recorded on the 1 February, 1971, for a sample from over the harbour spring. Nitrate was only occasionally present in the open water and then in trace quantities. It was therefore not included in Fig. 4.2. In the water over the harbour spring, the concentration of nitrate nitrogen was always high and fluctuated considerably during the year. There was a higher concentration of total organic nitrogen over the harbour spring than in the open water in March, June, September, 1970 and late February and March, 1971, but at other times this trend was reversed with higher concentrations of total organic nitrogen in the open water in April, May, November, December, 1970, and January and early February, 1971 (Fig. 4.2).

There was a significant correlation between total organic nitrogen and numbers of bacteria in the water over the harbour spring, and a highly significant correlation between total organic nitrogen and bacterial numbers in the open water (Table 4.1). However, fluctuations in nitrate nitrogen and the bacterial population of the water over the harbour spring were not correlated. No correlation coefficients involving soluble phosphate in both areas and nitrate nitrogen in the open water were calculated because the concentrations of these nutrients were usually so low.

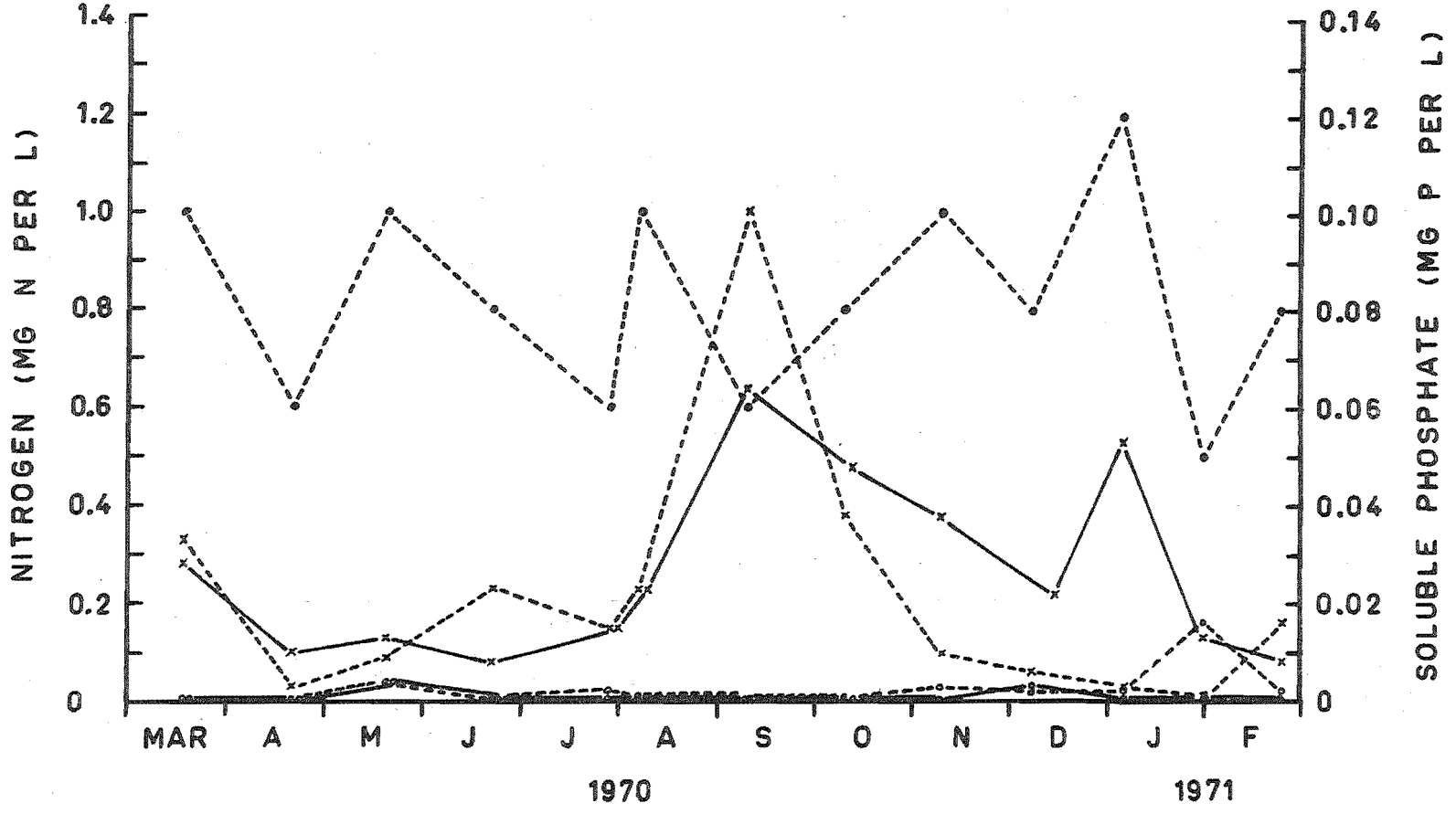


FIG. 4.2 Seasonal variation in concentrations of total organic nitrogen, nitrate nitrogen and soluble phosphate in lake water. Water over harbour spring: ----×---- total organic nitrogen; ---∘--- soluble phosphate; --- • --- nitrate nitrogen. Open water: ——×—— total organic nitrogen; ——∘—— soluble phosphate; nitrate nitrogen was never more than 0.05 mg/l.

Kinds of bacteria in the water over the harbour spring and in the main body of the lake

Bacteria were characterized in November, 1969, March, 1970, and April, 1970 (Table 4.3). Pseudomonads were found in all samples. In the samples characterized in November, 1969, flavobacteria and coryneforms were the main groups of bacteria found in addition to pseudomonads. Between the harbour spring (H) and open water samples collected in November, 1969, there were some differences. In the water over the harbour spring there were fewer coryneforms and flavobacteria, but Cytophaga was more common. The kinds of bacteria found in the other inlets will be considered later.

In March, 1970, samples from open water, over the weed and over the harbour spring were characterized. At this time the populations of bacteria in all three sites were similar. Apart from pseudomonads which made up a major part of the bacterial population of all the samples, Gram-negative fermentative rods, either Aeromonas/Vibrio or Enterobacteriaceae, were predominant. Flavobacteria and coryneforms were poorly represented in these samples. The only differences in the kinds of bacteria in these samples were that

i) the proportions of fermentative bacteria in the water over the harbour spring and water over the weed were lower than in the open water and

ii) fewer bacteria of the Alcaligenes/Achromobacter group were found in the open water.

In April, 1970, Gram-negative fermentative bacteria were still common (Table 4.3). The percentage of these bacteria was again lower in the sample from over the harbour

TABLE 4.3 Kinds of bacteria in Lake Grasmere and its inlets

Date sampled	Sample	No. characterized	Percentage distribution												
			Alcaligenes/ Achromobacter	pseudomonads	flavobacteria	Cytophaga	Vibrio extorquens	Enterobacteriaceae	Aeromonas/ Vibrio	Acinetobacter	Chromobacterium	coryneforms	Micrococcaceae	Bacillus
25/11/69	Open water	45	7	33	18	7		+	+			25	+	+
25/11/69	A	37	13	22	38				+			25		
25/11/69	E	47	+	27	21	+		17	6		+	19		
25/11/69	C	46	+	14	24		+		+	+	+	30	16	+
25/11/69	H	43	+	31	7	34		7	+			10	+	
17/ 3/70	Open water	51	+	23	+			51	20					
17/ 3/70	Over Elodea	47	11	32	+			34	11	+		+	+	+
17/ 3/70	H	49	17	36	+			25	14	+		+		+
20/ 4/70	Open water	48	31	21				33	15					
20/ 4/70	H	49	14	59	+			10	10			+		

+ ≤ 5%

spring compared with the open water sample.

The numbers of Enterobacteriaceae and Aeromonas/Vibrio per ml of water in the March and April samples are compared in Table 4.4. The numbers of these bacteria were similar in the samples of open water and water over weed, but there was some reduction in their numbers between March and April in the water over the harbour spring.

TABLE 4.4 Enterobacteriaceae and Aeromonas/Vibrio in water sampled in March and April, 1970

Date sampled (1970)	Sampling site	Enterobacteriaceae % (of those characterized)	Enterobacteriaceae no. (per ml)	Aeromonas/Vibrio % (of those characterized)	Aeromonas/Vibrio no. (per ml)
March	Open water	51	63	20	25
March	Water over weed	34	65	11	21
March	Water over harbour spring	25	28	14	16
April	Open water	33	68	15	31
April	Water over harbour spring	10	10	10	10

Bacterial populations of inlets A and C and stream E compared with those of the water over the harbour spring and the open water

As well as the harbour spring, three other inlets were sampled in November, 1969. The numbers of bacteria in inlets A and C were greater than the open water population (Table 4.5). The size of the population of stream E, the supposed source of water for A and C, was between that of A and the open water. In November there was only a moderate flow of water in E, which was then 0.7-1 m across and approximately 0.4 m deep. The large bacterial population in C was typical of this stream, but the other samples collected from C in 1971 also showed that the numbers of bacteria in this inlet could vary considerably (Table 4.5). The sample from over the harbour spring had the lowest number of bacteria of all the samples collected in November, 1969.

The kinds of bacteria found in the inlets in November, 1969, are shown in Table 4.3. Inlet C had a greater diversity of bacteria than the other samples, with 11 groups being represented although only four of these contained more than 5% of the isolates characterized. Micrococcaceae was one of the main groups of bacteria found in C but only single isolates were found in the other samples. Enterobacteriaceae were more common in the samples from E and over the harbour spring. The bacterial populations of spring A and the open water were similar in the main groups represented, but more groups of bacteria were found in the open water. It was noted on p.117 that the sample from over the harbour spring analyzed in November, 1969, was distinct from the open water

TABLE 4.5 Numbers of bacteria in inlets and open water

Date sampled	Rainfall on 7 days before sampling (cm)	No. bacteria (per ml)				
		Open water	H	A	C	E
25/11/69	2.11	200 ± 60	83 ± 28	850 ± 200	19 500 ± 9 600	410 ± 120
24/ 2/71	1.68	490 ± 180	570 ± 110		17 500 ± 6 300	
8/ 3/71	0.00	287 ± 84			1 600 ± 400	
22/ 3/71	1.02	256 ± 44			39 000 ± 9 000	

sample in the differing proportions of flavobacteria, coryneforms and Cytophaga. The proportions of these three groups in the samples from the other three inlets were also different from those of the harbour population.

Nutrient content of inlets and open water

Chemical analyses of the inlets A, C and E were not made on 25 November, 1969, but analyses for 11 November and 4 December, 1969, are shown in Table 4.6. The analyses of the open water and water over the harbour spring sampled on 25 November, 1969, and stream C sampled in 1971, are also included. The total organic nitrogen of E was very low in both samples analyzed. However, the concentration of total organic nitrogen in A, which was moderate on 11 November, 1969, had increased by 4 December, 1969. Of the 1969 samples, C had the highest concentration of total organic nitrogen - 0.5 mg N/l - on both 11 November and 4 December. The highest concentration of total organic nitrogen of all samples analyzed was also recorded in C on 22 March, 1971. Nitrate nitrogen was not found in E and the open water, and was present only as a trace in A. These amounts are not shown in Table 4.6. In C, the concentration of nitrate nitrogen was usually low. The highest concentration recorded in C was 0.02 mg/l on 24 February, 1971. In the water sampled from over the harbour spring inflow a high concentration of nitrate nitrogen was always recorded.

TABLE 4.6 Concentrations of total organic nitrogen, nitrate nitrogen and soluble phosphate in inlets and open water

Date sampled	Concentrations in mg N or P per litre											
	Open water[1]		HA				EA				CA	
	TON[1]	P[1]	TON	NO_3[1]	P	TON	P	TON	P	TON	P	NO_3
11/11/69	-	-	-	-	-	0.12	0.002	0.02	< 0.001	0.5	0.04	nil
25/11/69	0.11	0.003	0.2	0.8	0.003	-	-	-	-	-	-	-
4/12/69	-	-	-	-	-	0.38	0.004	0.01	< 0.001	0.5	< 0.001	nil
24/ 2/71	0.08	< 0.001	0.16	0.8	0.002	-	-	-	-	< 0.2	0.001	0.02
8/ 3/71	0.28	0.001	-	-	-	-	-	-	-	-	-	-
22/ 3/71	0.15	< 0.001	-	-	-	-	-	-	-	6.8	< 0.001	< 0.02

- no sample.

[1] TON - total organic nitrogen; NO_3 - nitrate nitrogen; P - soluble phosphate.

Metabolic activity of bacteria in open water and inlet C

The effect of inflow of water on bacteria other than those included in the plate counts could not be determined as no direct counts of bacteria were made when the inlets were sampled. Attempts to label bacteria in water samples and thus estimate the proportion which were metabolizing *in situ* were made, and produced some limited results (Table 4.7). In both the sample from C and the sample from the open water, only a small percentage of the total population formed an autoradiogram.

TABLE 4.7 Uptake of ^{3}H-glucose and ^{3}H-thymidine by bacteria in water samples, detected by autoradiography

Date sampled	Sampling site	Label	Exposure period (days)	Bacteria labelled (%)
24/2/71	C	^{3}H-glucose	13	9
24/2/71	C	^{3}H-thymidine	13	4
22/3/71	Open water	^{3}H-glucose	7	12[1]
22/3/71	Open water	^{3}H-thymidine	7	8[1]
22/3/71	Open water	^{3}H-thymidine	14	12[1]

[1] The bacteria of these samples had passed through a 10 μm filter and had been retained on a 0.45 μm filter.

4.3 DISCUSSION

Rainfall measurements

The amount of water flowing into a lake is regulated by the rainfall in the catchment area. Lake Grasmere is situated in a mountainous region and, although runoff is rapid (Hayward, 1967), some lag period is to be expected before any effect of rain on the lake bacteria is apparent. Much of the rain percolates through the ground before flowing into Lake Grasmere through springs. In this study the rainfall for 7 and 14 days before sampling was related to the numbers of bacteria in the water over the harbour spring. These data (not included) showed that between October, 1969, and March, 1971, the fluctuations in bacterial numbers in the water over the harbour spring were more closely related to the rainfall in the previous 7 days than to that in the previous 14 days. This may not always be true as during the study period, for 14 out of the 17 sampling dates considered, there was either no rain or less than 1.3 cm in the first half of the 14 days before sampling. Because there was often such a low rainfall, the rainfall in the 7 days immediately before sampling provided the most satisfactory measure of inflow of water.

Relationship of bacteria in the water over the harbour spring to rainfall and nutrient concentrations

The numbers of bacteria in the water over the harbour spring were generally low and of a similar order of magnitude to the population in the main body of the lake. This

suggested that large numbers of bacteria were not usually brought into the lake through this spring. In addition, the kinds of bacteria in the harbour samples did not suggest an inflow of bacteria normally found in soil. In the three samples from over the harbour spring analyzed, large proportions of coryneforms and spore-forming bacteria, which frequently make up to 80% of the soil bacterial population (Burges, 1965) were not found.

As the harbour spring was only occasionally a source of bacteria, it appeared that the numbers of bacteria in the water over the spring were controlled by the inflow of nutrients throughout most of the year. At these times the effect of increased flow of water into the harbour was, as was concluded by Taylor (1949), to induce growth and multiplication of the harbour bacteria in response to the inflow of nutrients. Thus, it is likely that the correlation coefficient between rainfall and numbers of bacteria in the water over the harbour spring was highly significant because rainfall and nutrient inflow could often be equated.

While the fluctuations in the numbers of bacteria in the water over the harbour spring between April and August, 1970, appeared to be directly proportional to the amount of rainfall before sampling, at other times of the year the size of the fluctuation in the bacterial population did not correspond with the amount of rainfall. In September, 1970, and January, 1971, numbers of bacteria were higher, and in November, 1970, lower, than might have been expected from the amount of rainfall in the 7 days before sampling (Fig. 4.1). The large numbers of bacteria in the water over the harbour spring in September, 1970, suggested that many bacteria had

been washed into the lake. As the harbour was very turbid in September, 1970, it seemed likely that the very large population was made up mainly of bacteria attached to silt as noted by Collins and Willoughby (1962) after heavy rain, and Fondén (1969a) after the melting of snow. This increase was short-lived and by 9 October, 1970, a population of 480 bacteria per ml was found in the water over the harbour spring.

The peak in the harbour spring bacterial population at the beginning of January, 1971, followed only a moderate rainfall. However, the nitrate concentration at this time was very high and may have been responsible for the increase in numbers of bacteria. The small bacterial population recorded in November, 1970, suggested that the nutrient content of the inflowing water was lower than usual. The very heavy winter rains which fell in 1970 may have leached the ground to such an extent that the effect of 4.2 cm of rain at the beginning of November was to dilute a small supply of nutrients and reduce numbers of bacteria in the water over the harbour spring.

Thus, the inflow of nutrients appeared to be the main factor influencing the numbers of bacteria in the water over the harbour spring except at certain times, as in September, 1970, when the washing-in of bacteria masked the effect of any addition of nutrients. Rainfall, while significantly correlated with numbers of bacteria in the water over the harbour spring was only one of several factors controlling this inflow of nutrients. The influence of factors, other than rainfall, on nutrient inflow was emphasized by the unexpected responses by the bacteria in November, 1970 and January, 1971.

Nutrients, in this context, are any available organic matter and also any elements, such as phosphorus, which may be limiting growth of the bacteria. The determination of total organic nitrogen provided a measure of all nitrogen associated with organic matter and, thus, also gave a relative indication of the concentration of organic matter. This parameter included both living organisms and detritus, some of which would not be 'available nutrients' for bacteria. The nitrate determinations indicated the concentration of soluble nitrogen being brought into the lake.

It was found that total organic nitrogen and numbers of bacteria in the water over the harbour spring were significantly correlated although the correlation was not as close as that between rainfall and numbers of bacteria over the spring (Table 4.1). Total organic nitrogen would have been at times a measure of inflowing nutrients as, for example, in September, 1970, when silt flowed into the lake, but would have been more often a measure of the response of other organisms, such as algae, to the addition of nutrients. Thus, although total organic nitrogen and numbers of bacteria in the water over the harbour spring were significantly correlated this did not necessarily mean that the size of the bacterial population was directly related to the concentration of total organic nitrogen. For example, in January, 1971, there was a peak in bacterial numbers although the concentration of total organic nitrogen was low.

The bacteria in the water over the harbour spring did not respond to the consistently high concentration of nitrate nitrogen recorded in the harbour samples. Phosphate might

have been limiting growth of bacteria as phosphate concentrations in the water over the harbour spring were often too low to be detected, but with the data available only speculation on the role of phosphate is possible. The increase in numbers of bacteria in late February may have been related to the unusually high concentration of phosphate recorded on 1 February, 1971.

Thus a simple estimate of the nutrients to which the bacteria in the water over the harbour spring were responding could not be obtained by determination of total organic nitrogen, nitrate nitrogen or soluble phosphate. Chemical determinations give a measure of the potential nutrients but the responses of the bacteria near the harbour spring were for most of the year the best indication of the quantities of nutrients available for recycling. Further studies combining estimates of bacterial numbers in the water over the harbour spring and a number of elements would help to determine which nutrients essential for bacterial growth were being provided through this inlet.

The bacterial population of the main body of the lake

While rainfall and numbers of bacteria in the water over the harbour spring were significantly correlated, a similar correlation was not found in the main body of the lake. However, the large increase in the harbour bacterial population in September, 1970, attributed mainly to a washing-in of silt and bacteria, was also apparent in the open water. The data from Elodea and water over Elodea sampled at this time also suggested that a large proportion of this increased

bacterial population was attached to particulate matter. The numbers of bacteria in the water over the weed were smaller than in the open water, but the bacterial population on the Elodea was much larger than had been found in the previous two months (Fig. 6.1). It appeared that silt and attached bacteria had sedimented on to the weed and this had resulted in a lower number of bacteria in the water over the weed. This sedimentation effect may have been less apparent in the open water because it was sampled at 3 m instead of 1.75-2 m, which was the sampling depth for the water over the Elodea beds. In addition, silt would have been more readily circulated from one depth to another in the open water than among the Elodea beds.

At other times of the year increases in the numbers of bacteria in response to an inflow of nutrients, as noted in the harbour in July, 1970, could not be detected in the main body of the lake. This lack of a relationship could have been due to a rapid utilization of nutrients near the inlets and dilution by the main body of the lake, as suggested by the low nitrate concentrations in the open water compared to the water over the harbour spring. Also, the growth and death of other organisms would be affecting bacteria and may have masked the effect of any inflow of nutrients. Although rainfall and numbers of bacteria in the open water were not correlated, total organic nitrogen and numbers of bacteria were.

However, the importance of rainfall as a source of nutrients in the open water of Lake Grasmere was suggested by the data in Table 4.2. Numbers of bacteria were lowest

between March and June when low rainfall in the previous winter and spring months was combined with a low rainfall before sampling, as occurred in 1969/1970. It appeared that nutrients were limiting bacterial multiplication after the low rainfall between August and November, 1969, and this situation was not alleviated by the higher than usual rainfall recorded between December, 1969, and February, 1970. In 1971, although rainfall was low before sampling and between December, 1970, and February, 1971, numbers of bacteria were generally higher from March to June, 1971, than during the same period in 1970.

The rainfall during the previous winter and beginning of the new growing season appeared to be a critical factor in the control of numbers of bacteria at the end of the growing season. Whereas the bacterial population in the harbour could be analyzed on the basis of recent events, in the open water events over a longer period needed to be taken into account. The main effects of an increased inflow of water and nutrients into the lake over a long period would have been to increase the supply of nutrients to the primary producers, when the amount of substrate available for bacterial decomposition would increase, and also to provide nutrients essential for the metabolism of bacteria so that they could utilize any organic matter present.

Kinds of bacteria in the water over the harbour spring and in the main body of the lake

The different proportions of flavobacteria, coryneforms and *Cytophaga* in the samples collected in November, 1969, from

the harbour and open water (Table 4.3) suggested that the nature of the nutrients in the two areas was not the same, although the numbers of viable bacteria in both samples were similar. The Enterobacteriaceae in the water over the harbour spring had probably originated from stock which often drink from the harbour.

The March and April, 1970, samples were unusual when compared with all the other samples characterized. They contained large proportions of fermentative bacteria - Aeromonas/Vibrio and Enterobacteriaceae. In both March and April very little rain fell in the 7 days before sampling so a large inflow of these bacteria into the lake was unlikely to have been the cause of their abundance. In the harbour, stock may have been the main source of these bacteria. The drought conditions may have attracted the stock to the harbour more often than usual thus resulting in a higher proportion of Enterobacteriaceae in the water over the harbour spring than was found in the November sample from over the spring. However, the abundance of both Enterobacteriaceae and Aeromonas/Vibrio in the main body of the lake was unexpected. Calculation of the numbers of these bacteria per ml of water showed that their numbers were remarkably constant in the samples of open water and water over weed although the total numbers of bacteria per ml varied (Table 4.4).

The possibility that waterfowl were the main source of these bacteria was considered. Paradise duck, Canada geese and black swans are the birds most commonly found on the lake. The shooting season extends from the beginning of May to the beginning of June for Paradise duck and until the end of July for the geese and swans. As Lake Grasmere

is a bird sanctuary, the numbers of birds on the lake increase markedly from March each year but decrease again by June or July. The similar numbers of fermentative bacteria in March and April in the main body of the lake could have been due to a continual addition of these bacteria by the waterfowl. The presence of these bacteria was, however, somewhat of an enigma. They were most likely to be associated with the faeces of the waterfowl so that a corresponding increase in total organic nitrogen would be expected. Yet although total organic nitrogen in the open water was high in March it was low in April. Furthermore, the addition of organic matter in such quantities would influence the total numbers of bacteria, but numbers of bacteria were low throughout March and April. In April of the previous year none of these bacteria were found in the open water although Enterobacteriaceae made up 4% (equivalent to 44 bacteria per ml at this time) of the bacteria isolated from the sample of water over weed.

However, waterfowl were still the most likely source of the fermentative bacteria whose predominance in 1970 to the near exclusion of flavobacteria and coryneforms may have been related to the generally low numbers of bacteria in the lake that year. The total numbers of bacteria in the open water were higher in 1969 (Table 4.2). Possibly faeces from the waterfowl, which would include crystals of water-insoluble uric acid, sedimented out rapidly so that little increase in total organic nitrogen was detected in the lake water.

These 1970 water samples were particularly interesting as an example of the complex interaction of factors influencing bacterial populations in water. It is possible that

the low rainfall experienced in the previous winter and during the sampling period culminated in a low concentration of essential nutrients in the lake. At the same time birds were attracted to the lake in greater numbers at the end of the summer because of drought conditions. The result was a predominance of 'foreign' bacteria in the lake but the lack of essential nutrients did not encourage multiplication of the lake microflora. Thus, total numbers of bacteria were low and some of the kinds of bacteria usually making up a part of the aquatic bacterial population were absent.

Bacterial populations of inlets A and C and stream E compared with those of the water over the harbour spring and the open water

The three inlets A, C and E were sampled together with the harbour spring and open water only once in November, 1969. Stream C had a larger bacterial population and higher concentrations of organic nitrogen and phosphate than the other inlets. The high concentration of phosphate in stream C on 11 November may have been the cause of the measurable amount of phosphate in the open water on 25 November.

Increases in the numbers of bacteria in the water over the harbour spring have been mainly attributed to the multiplication of bacteria. The flow of water through C was much greater than through the harbour spring. As C was often silty and a larger proportion of Gram-positive bacteria was found, it seemed likely that washing-in of soil particles with attached bacteria was responsible for the larger bacterial population of this inlet. After the moderate rainfall in November, numbers of bacteria were very low in the water over

the harbour spring but this rainfall was sufficient to wash large numbers of bacteria into C. The numbers of bacteria in the open water on 25 November, 1969, were low (200 bacteria per ml), but this bacterial population was twice that in the water over the harbour spring in November and in the open water in October, 1969. The increase in the numbers of bacteria in November in the open water could have been due to the addition of bacteria from C combined with some induction of growth of bacteria in response to the addition of phosphate earlier in November, 1969.

Stream E and spring A were sampled so that the numbers and kinds of bacteria in water before and after it had percolated through a shingle fan could be compared. The bacterial population of A was twice that of E. Either nutrients and bacteria were leached from the ground as the water flowed from E to A or there was an increase in numbers of bacteria at site A, possibly because of the cattle drinking at the spring. No Enterobacteriaceae were found in the sample from A and Gram-positive bacteria did not make up a major part of the bacterial flora. It seemed likely that the higher bacterial population was due to some additional nutrients rather than bacteria and that this spring would not normally be a source of large numbers of soil bacteria. The lack of Enterobacteriaceae in A was interesting as this group made up 17% of the population of E. They might have been found in A at a late date or percolation through a shingle fan may have eliminated these bacteria. The relationship of the Enterobacteriaceae in E to Escherichia coli is considered in the next chapter. Apart from the presence of Enterobacteriaceae in E the bacterial populations of E and A were similar.

The numbers of bacteria in C varied considerably as seen from the samples taken in February and March, 1971, (Table 4.5). Rainfall was not the only factor controlling the bacterial population of the stream. In November, 1969, after 2.1 cm of rain before sampling there were 19 500 bacteria per ml, but on 22 March, 1971, a much higher population was found after 1.0 cm of rain. This large bacterial population was probably related to the very high concentration of total organic nitrogen which was recorded in the stream at this time. Although the numbers of bacteria in C fluctuated in February and March there was no corresponding change in the numbers of bacteria in the open water.

The comparison of the bacterial populations of A, C, E and H showed that rainfall did not have the same effect on the numbers of bacteria in the various inlets. However, in general the inlets all appeared to have little direct effect on the open water population. It is likely that an immediate increase in the numbers of bacteria in the open water, which could be attributed to an increased flow of water through the inlets, would only be found after a period of very heavy rain.

Metabolic activity of bacteria in open water and inlet C

The experiments in which lake and stream bacteria were labelled with ^{3}H-glucose and ^{3}H-thymidine were not extensive enough for many conclusions to be drawn. Difficulties in concentrating the bacteria and ensuring a low background level of grains meant that only a few reliable counts were made (Table 4.7). These counts provided a measure of the percentage of the population which was metabolizing when the

samples were collected. On 24 February, 1971, only 9% of the stream bacteria took up radioactive glucose. The difficulties in interpreting the thymidine uptake data were discussed on pp. 73-76 and, from the results obtained, it can only be said that the proportion of the population which was dividing was very small. The glucose and thymidine results showed that apparently the stream milieu was not suitable for metabolism of the stream bacteria. The lack of active growth of the majority of the washed-in bacteria would support the hypothesis of Taylor (1949) that there is a rapid death of many washed-in bacteria once they reach a lake. In the open water of the lake, a sample collected on 22 March, 1971, had a larger proportion of metabolizing bacteria than the stream sample. The results of uptake of ^{3}H-glucose are given for an exposure period of 7 days only. As shown in Chapter 2, maximum uptake will not necessarily have been detected in this time. Taking into account the thymidine results after 14 days exposure, the real percentage of the population taking up glucose would probably have been about 16-18%. These results were interesting but unfortunately no direct comparison between the stream and open water populations was available.

This study of the relationship between rainfall and the bacteria of Lake Grasmere showed that the amount of rainfall was an important factor affecting the fluctuations of bacterial populations. A continual inflow of water and nutrients was essential if numbers of bacteria in the open water were to be maintained. However, it was impossible to generalize on the effects of rainfall. There was not

always a direct relationship between amount of rainfall and the concentration of nutrients flowing into the lake. The nature of the inlet determined whether its main contribution was nutrients or bacteria and the size of the bacterial population within the lake influenced the effect of a moderate addition of bacteria or nutrients. While the numbers of bacteria in the water over the harbour spring often provided a measure of the nutrients entering the lake at this point, the response of these bacteria could not be extrapolated to the whole lake because the effect of additional nutrients was often masked by such factors as the level of production of organic matter in the lake and, possibly, the addition of bacteria through C.

The question of the nature of the nutrients brought into the lake has not been considered in detail. In both the harbour and the open water, numbers of bacteria were significantly correlated with total organic nitrogen, and presumably, the amount of organic matter. However, the low numbers of bacteria in March and April, 1970, when it was thought much organic matter was being added by the waterfowl, suggested that the quantity of organic matter in the water was not a factor limiting numbers of bacteria. The low concentration of phosphate may be one of the causes of the low bacterial population of Lake Grasmere. Nitrate nitrogen, although at a consistently high concentration over the harbour spring, was not associated with a bacterial population significantly larger than that in the open water.

The techniques used to determine the proportion of metabolizing bacteria could be expanded in further experiments. A study of the proportion of metabolizing bacteria

and the concentrations of certain nutrients in the open water and inlets at different times of the year might help to determine which nutrients are limiting bacterial activity in Lake Grasmere.

4.4 SUMMARY

1) Numbers of bacteria in the main body of the lake were not directly influenced by rainfall in the 7 days before sampling unless it was very heavy. However, a low rainfall during the winter months appeared to be one factor controlling the size of the bacterial population in the open water of the lake in the following autumn.

2) Numbers of bacteria over the harbour spring were significantly correlated with amount of rainfall in the 7 days before sampling. However, this population was not much greater than that in the open water, suggesting that large numbers of bacteria were not washed into the lake through this inlet.

3) In both the open water and the water over the harbour spring, there was a significant correlation between numbers of bacteria and total organic nitrogen. No correlation between nitrate nitrogen and harbour bacteria was found. Concentrations of phosphate in both areas, and nitrate in the open water, were too low to calculate correlation coefficients.

4) Less than 20% of the bacterial populations of the open water and stream C formed autoradiograms when labelled with ^{3}H-glucose or ^{3}H-thymidine.

5) When several inlets were sampled at the same time, similar kinds of bacteria were found in stream E, spring A and the open water but, in stream C, there was a larger proportion of Gram-positive bacteria suggesting an inflow of soil. Over the harbour spring, more Cytophaga were found.

6) A combination of low rainfall in the winter of 1969 and the presence of many waterfowl on the lake in the autumn of 1970 was suggested as the reason for the predominance of Enterobacteriaceae throughout the lake in March and April, 1970.

CHAPTER 5

THE ECOLOGY OF ENTEROBACTERIACEAE ISOLATED FROM LAKE GRASMERE AND ITS INLETS

Many of the species of the family Enterobacteriaceae normally inhabit the intestine of mammals and other animals. The general term 'coliform' is frequently applied to these enteric bacteria. This term may be confined to enteric bacteria that can ferment lactose or may include all the enteric bacteria of the Enterobacteriaceae (Cowan, 1968). In the present study the term 'coliform' is applied only to those Enterobacteriaceae which can ferment lactose within 24-48 h.

The Enterobacteriaceae are characterized by being Gram-negative and oxidase-negative and having a fermentative utilization of glucose (Carpenter, Lapage and Steel, 1966). Within the family there are a number of genera which are differentiated with biochemical tests. However, although some bacteria give well-defined results to these tests there are others which give positive results only after an extended incubation period. Further difficulties in identification may arise if strains give some results typical of one genus and some typical of another (Parr, 1939).

In aquatic bacteriology, the Enterobacteriaceae are of particular interest as the presence of certain strains in water is an indication of faecal contamination. As some of the species of the Enterobacteriaceae are pathogenic and their most common method of transmission from one animal to

another is via water supplies, many domestic water supplies are routinely tested for the presence of indicator species such as Escherichia coli. Escherichia is usually distinguished from some of the other genera of the Enterobacteriaceae by its ability to ferment lactose with the formation of gas within 24 h at 44°C, and from other lactose-fermenting genera on the basis of the results of tests such as methyl red, Voges-Proskauer and citrate utilization (Carpenter, Lapage and Steel, 1966).

In some of the samples from this study, there were a large number of bacteria which were identified as Enterobacteriaceae. This suggested there might be, on occasions, gross faecal contamination of the lake. The nearest human habitation to Lake Grasmere is Grasmere Station. This is about 2 km from the lake and there is no direct flow of water from the Station to the lake. Towards the south end of the lake there is a privy about 30 m from the lake which is used by fishermen and campers. This is unlikely to be a major source of contamination. Other sources of coliforms could be cattle and sheep which graze on some of the surrounding land, and waterfowl which are found on the lake in large numbers at certain times of the year.

It is possible that the Enterobacteriaceae isolated from Lake Grasmere may not have been typical coliforms. Among the coliforms from lakes and streams studied by Taylor (1941) were a number which gave inconclusive results to the biochemical tests used. Genera of the Enterobacteriaceae which are widely present in soil and water are Enterobacter and Serratia (Stanier, Douderoff and Adelberg, 1970). Collins (1970) isolated Enterobacter and Proteus as well as

which, however, received sewage effluent from an activated sludge plant of a small town. Escherichia from an oligotrophic lake. Enterobacteriaceae, which were designated Enterobacter cloacae, have been found on plants (Kroulik, Burkey and Wiseman, 1955; Stout, 1960).

In this chapter the identity of the Enterobacteriaceae isolated from Lake Grasmere and its inlets and their distribution within the lake are considered. The ability of isolates to ferment lactose was tested and further differential tests, mostly described by Harrigan and McCance (1966), were applied to a selection of the isolates.

5.1 METHODS

During this study, 129 of the bacteria routinely isolated for characterization from different habitats within the lake and from the inlets were identified as members of the family Enterobacteriaceae. The habitat of each isolate was recorded and 98 were tested further so that their similarity to the faecal coliform Escherichia coli could be determined. Two isolates tentatively identified as Enterobacteriaceae and one identified as Aeromonas were also included in the series for comparison.

Procedure to check purity of stock cultures

Details of the methods and media used are described on pp. 34-35. Incubation was at 26°C. Isolates were subcultured to nutrient broth from stock cultures maintained under mineral oil and were incubated for 24 h. This broth culture (1) was used to inoculate:-

a) nutrient broth (2) - the morphology of the cells

was recorded after 24 h incubation. The cells were stained with safranin;

b) GYCA - the appearance and purity of the colonies was noted after incubation for 3 - 4 days. The presence of oxidase was also tested;

c) one bottle of Hugh and Leifson medium - this was examined after 1 and 4 days for acid production.

Tests

Details of the methods and media used are described on p.45 except when page nos. are specified.

Fermentation of lactose: Using broth culture (1), all isolates were inoculated into lactose broth. All bottles were examined for acid and gas formation after 1 and 4 days. Some were also examined after 2 days.

Thirty-eight isolates, which included the three not identified conclusively as Enterobacteriaceae, were tested further. Most of the following tests were carried out on each isolate:-

Fermentation of glucose, inositol and salicin,

MR and VP tests,

Citrate utilization,

Urease production,

Phenylalanine deaminase production,

Gelatin liquefaction,

H_2S production.

The following tests were carried out on some of the 101 isolates:-

Production of ammonia from arginine: Forty-seven isolates were tested as described on p. 36.

Flagellation: The flagella of 34 of the 38 isolates tested in detail, as well as 8 additional isolates, were examined under an EM as described on p. 34.

Fermentation of lactose in McConkey broth incubated at 30°C: Thirty-two of the isolates chosen for further testing were examined.

5.2 RESULTS

Apart from the samples collected in March and April, 1970, and from stream E on one occasion, Enterobacteriaceae rarely made up a major part of the bacterial flora of the samples from Lake Grasmere and its inlets (Table 5.1). However 16% of the isolates from stream E, which was sampled in November, 1969, were Enterobacteriaceae, and, in March and April, 1970, a large proportion of the isolates in water sampled from three different areas of the lake were Enterobacteriaceae.

Among all the strains of Enterobacteriaceae isolated during this study were 21 which were non-motile. These were not tested further as they were not initially keyed out as Enterobacteriaceae. They were isolated from the following samples:-

25 November, 1969: open water - 5; stream E - 1; over harbour spring - 3;

17 March, 1970 : open water - 4; over harbour spring - 1; over weed - 3;

TABLE 5.1 Origin of Enterobacteriaceae isolates

Sample	No. characterized	No. Enterobacteriaceae	% Enterobacteriaceae
Open water [1]	672	18	3
Open water-March, April, 1970	99	44	44
Over weed [1]	365	12	3
Over weed-March, 1970	47	14	30
Over harbour spring [1]	48	3	6
Over harbour spring-March, April, 1970	98	14	14
Stream E	51	8	16
Inlet C	90	3	3
Inlet A	37	0	0
Elodea canadensis	260	11	4
Mud	167	2	1
Plankton	155	0	0
Total	2089	129	6

[1] All samples except those collected in March or April, 1970

20 April, 1970 : over harbour spring - 5.

Procedure to check purity of stock cultures

Nutrient broth smears: Many of the isolates had none or only a few of the short cells typical of Escherichia.

GYCA: All isolates appeared to be pure but many did not grow so abundantly and were cream-coloured instead of white as originally recorded.

Oxidase: All but four of the isolates were oxidase-negative, One of the positive cultures had been originally identified as Aeromonas and included in the study for comparison. The remaining three isolates fermented glucose rapidly and two were found to have peritrichous flagella. The flagella of the third isolate were not examined.

Glucose fermentation in medium of Hugh and Leifson: All except five isolates fermented glucose after 1 day and these five isolates were positive after incubation for 4 days. The five isolates included the two isolates only tentatively identified as Enterobacteriaceae. These were found to have polar flagella. The other three isolates had peritrichous flagella.

Lactose fermentation of all isolates

Of the 14 isolates which fermented lactose within 24 h (Table 5.2), only two did so with the formation of gas. The majority fell into the group of slow lactose fermenters. However, there was a predominance of isolates which could not ferment lactose in the sample of Elodea collected in 1969.

TABLE 5.2 Fermentation of lactose at 37°C

Date sampled	Sample	No. isolates giving reaction			Total
		Lactose fermented (after 1 day)	Lactose fermented (after 4 days)	Lactose not fermented (after 4 days)	
15/ 4/69	Over weed	0	7	1	8
15/ 4/69	Mud	0	1	1	2
17/ 6/69	Open water	0	3	1	4
17/ 6/69	Over weed	0	1	0	1
28/10/69	*Elodea*	1	0	8	9
28/10/69	Over weed	0	1	0	1
11/11/69	Open water	1	1	1	3
11/11/69	C	1	2	0	3
25/11/69	Open water	2	3	0	5
25/11/69	E	1	2	4	7
17/ 3/70	Open water	1	18	1	20
17/ 3/70	Over weed	0	10	1	11
17/ 3/70	Over spring H	2	2	2	6
17/ 3/70	*Elodea*	0	1	0	1
20/ 4/70	Open water	5	12	0	17
7/12/70	Open water[1]	0	0	1	1
13/ 7/71	Mud[1]	0	0	1	1
13/ 7/71	*Elodea*[1]	0	0	1	1
Total		14	64	23	101

[1] Bacteria included for comparison.

Additional tests on selected isolates

The ability of 35 isolates to ferment lactose at 30°C compared to that at 37°C was examined (Table 5.3). The three isolates included for comparison were not tested in this way. Of the eight isolates which did not ferment lactose at 37°C after incubation for 4 days, only three, which were from Elodea, were able to ferment lactose a little after 4 days at 30°C. Lowering the temperature of incubation of the remaining 27 isolates which could ferment lactose at 37°C resulted in a more rapid fermentation of lactose by only six isolates. Of these, three isolates were positive after incubation for 3 days at 30°C. (Isolates were not examined after 3 days incubation at 37°C). Eleven isolates, which did not produce gas at 37°C did so at 30°C. On the other hand there were four isolates which produced gas only at 37°C. However, in all cases, gas did not fill more than 10% of the inverted tube even after incubation for 4 days.

Thus, few isolates could ferment lactose vigorously at either temperature. Half of the isolates were also unable to produce gas from glucose or did so only slowly (Table 5.3).

The results of some of the other tests (Appendix 3) were also difficult to interpret. The MR and VP tests were carried out twice on most of the cultures. After incubating for 2 days at 26°C, some isolates were MR+ and VP+ and some were MR- and VP-. Incubation for 4 days at 30°C reversed the reactions of two isolates, and five in which the MR and VP reactions had been the same were differentiated. However, three isolates were still definitely both MR+ and VP+ (Appendix 3, B and C).

TABLE 5.3 Rate of fermentation of glucose at 37°C and lactose at 30°C and 37°C by selected isolates

date sampled	Isolate source	no.	No.days incubation before positive test: lactose fermentation 30°C (McConkey broth) acid	gas	lactose fermentation 37°C (lactose broth) acid	gas	Gas from glucose
15/ 4/69	Over weed	54	4	4	2	4	4
15/ 4/69	Over weed	120	2	3	2	-	1
15/ 4/69	Over weed	133	3	-	2	4	1
15/ 4/69	Over weed	195	3	2	2	-	1
15/ 4/69	Over weed	210	3	3	4	-	1
15/ 4/69	Mud	369	NT	NT	2	-	-
15/ 4/69	Mud	410	-	-	-	-	-
17/ 6/69	Open water	4	4	-	4	-	-
17/ 6/69	Open water	20	2	4	2	-	4
17/ 6/69	Over weed	52	NT	NT	4	-	1
28/10/69	Elodea	2	sl 4	-	-	-	2
28/10/69	Elodea	11	sl 4	-	-	-	2
28/10/69	Elodea	41	4	3	-	-	1
28/10/69	Over weed	3	3	3	4	-	1
11/11/69	Open water	38	-	-	-	-	2
11/11/69	C	1	-	-	4	-	-
11/11/69	C	15	-	-	1	-	4
11/11/69	C	44	-	-	4	-	-
25/11/69	E	1	3	3	2	-	-
25/11/69	E	5	3	-	2	4	-
25/11/69	E	48	2	-	1	2	-
25/11/69	Open water	38	2	-	1	2	2
17/ 3/70	Open water	3	-	-	-	-	-
17/ 3/70	Over weed	14	NT	NT	4	-	1
17/ 3/70	Over weed	20	-	-	-	-	-
17/ 3/70	Over weed	26	3	4	2	2	NT
17/ 3/70	Elodea	44	2	2	4	-	-
17/ 3/70	Over spring H	47	-	-	-	-	1
20/ 4/70	Open water	2	2	2	4	-	1
20/ 4/70	Open water	3	2	2	1	1	NT
20/ 4/70	Open water	12	2	3	1	2	NT
20/ 4/70	Open water	22	2	4	2	2	NT
20/ 4/70	Open water	23	2	3	4	-	1
20/ 4/70	Open water	35	2	2	4	-	1
20/ 4/70	Open water	38	2	3	1	1	NT

\- negative reaction after incubation for 4 days.

sl slight positive.

NT not tested.

Inositol fermentation in Appendix 3 was recorded after 2 days at 37°C but results obtained after incubation for 4 days, indicated that some isolates which were negative after 2 days, were capable of fermenting inositol if the incubation period was extended. None of the isolates tested produced phenylalanine deaminase. The tests for citrate utilization, gelatin liquefaction and H_2S formation gave well-defined results. The majority of isolates were unable to liquefy gelatin but those that could were found in all three groups (A, B and C, Appendix 3). Only four isolates could produce H_2S and these were all in group C. However, isolates that could, and could not, utilize citrate were found in all three groups.

Flagella were peritrichous (Fig. 5.1) in all but two of the isolates examined (Appendix 3). In addition to the isolates recorded in Appendix 3 eight more were examined and all had some cells with peritrichous flagella. However, four isolates from Elodea had only occasional cells with peritrichous flagella; the remainder having one or two subpolar flagella.

Identity of isolates selected for further study

On the basis of the results of lactose fermentation, MR and VP tests, citrate utilization and urease formation, isolates were grouped into the genus with which they had most in common (Appendix 3). Five genera were recognized. Escherichia, Salmonella, Enterobacter and Proteus were differentiated using the characters described by Davis et al. (1969) and Skerman (1967). The characters noted by Stanier, Douderoff and Adelberg (1970) were used for the differentiation

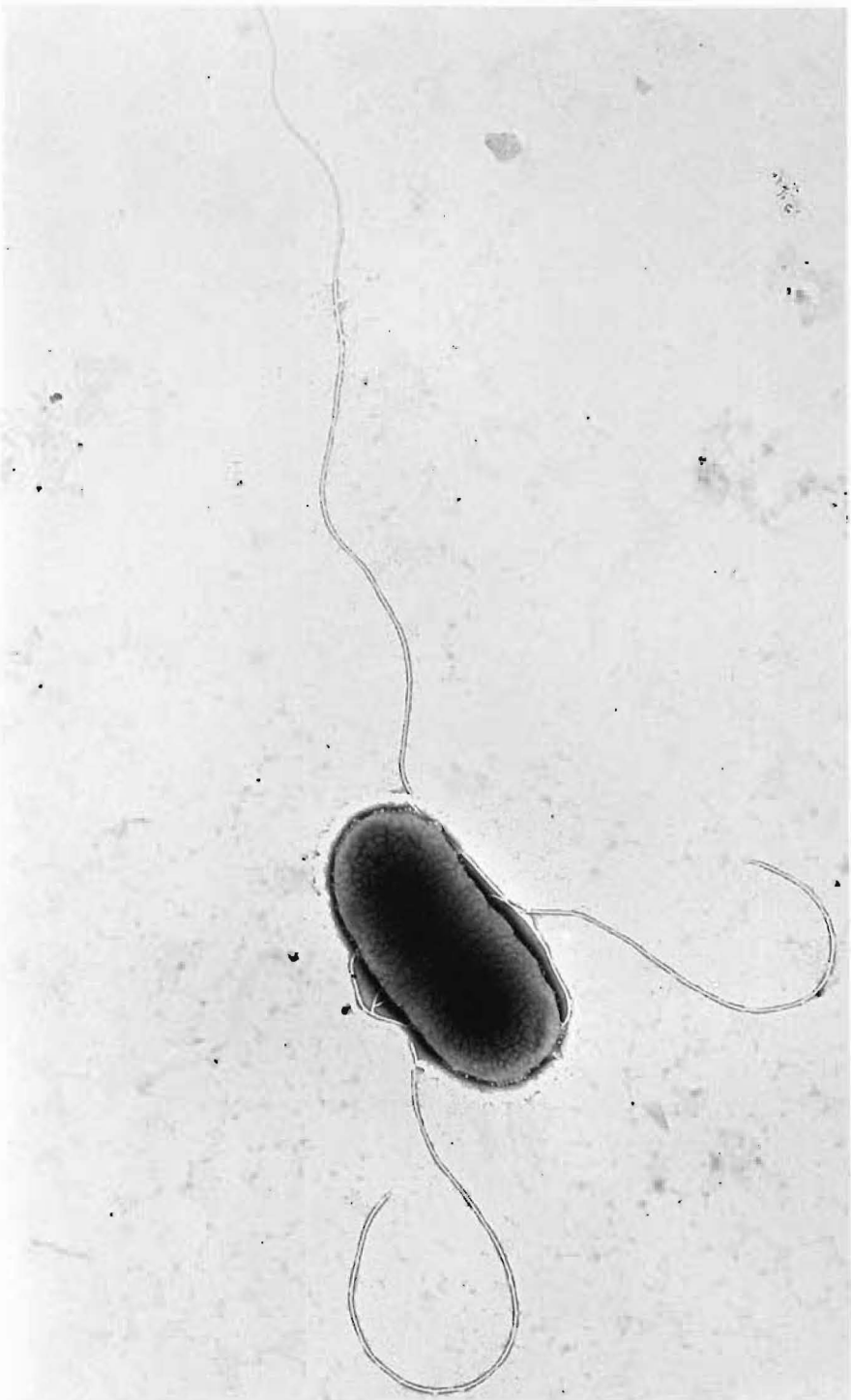


FIG. 5.1 Electron micrograph of Enterobacteriaceae isolate, showing peritrichous flagella; x 18 500.

of Serratia.

Table 5.4 shows the distribution among these genera of the isolates on which tests in addition to lactose fermentation were carried out. The similarity of isolates to the genus to which they were allocated varied.

Enterobacter: 18 isolates were grouped as Enterobacter although nine could not utilize citrate. This genus was found in many of the samples which included Enterobacteriaceae isolates and, as far as can be concluded from the limited number of isolates examined in detail and from the lactose fermentation of the remainder, was the predominant genus of the Enterobacteriaceae in the March and April, 1970, samples.

Proteus: This genus comprised mainly isolates which could not ferment lactose. The six Proteus-like isolates did not have spreading colonies typical of Proteus and the MR and VP tests varied between isolates, but all isolates were urease-positive. The presence of urease was the main character distinguishing these bacteria from Salmonella. Proteus was not found in the samples collected in April, June and October, 1969, but there were one or two representatives in most of the other samples including streams C and E.

Salmonella: Six isolates were grouped as Salmonella. Two of these isolates were able to ferment lactose slowly but were differentiated from Escherichia because they were able to utilize citrate. The other four isolates could either not ferment lactose or do so only slowly at 30°C.

TABLE 5.4 Distribution of genera in samples from Lake Grasmere and its inlets

Sample	No. of isolates in each genus (tentative identification)							
	Proteus	Salmonella	Escherichia	Enterobacter	Serratia	Aeromonas[1]	Vibrio[1]	Total
Open water	2	2	1	7	0	0	1	13
Over weed	1	1	0	8	0	0	0	10
Elodea	0	3	0	1	0	1	0	5
Mud	0	0	1	0	1	1	0	3
C	2	0	0	1	0	0	0	3
E	1	0	2	0	0	0	0	3
Over spring H	0	0	0	1	0	0	0	1
Total	6	6	4	18	1	2	1	38

[1] Only three isolates from these genera were examined in this study.

These isolates also did not utilize citrate. Thus, none of these isolates was identical to Salmonella. Three of the Salmonella-like isolates were from Elodea.

Escherichia: Four isolates from mud, open water and stream E were grouped as Escherichia. Three of these could utilize citrate but because they fermented lactose rapidly, two with the formation of gas within 48 h, were grouped as Escherichia. These three were isolated at the same time in November, 1969.

Serratia: One isolate from the mud was grouped as Serratia. Although it did not produce a red pigment, it was not able to produce gas from glucose and lacked the ability to ferment lactose. The results of the MR and VP tests differentiated it from Salmonella and the lack of urease from Proteus.

Three isolates were not allocated to any genus of the Enterobacteriaceae. Two of the isolates included in the group for detailed study had only been tentatively identified as Enterobacteriaceae because their fermentation of glucose in the medium of Hugh and Leifson was not rapid. One other had originally been identified as Aeromonas as it was oxidase-positive. Examination of the flagella of the two possible Enterobacteriaceae showed that they both had polar flagella. One of these - July, 1971, Elodea no. 50 (27Gr 50) - also liquefied gelatin, utilized citrate, produced H_2S and could produce ammonia from arginine (Appendix 3,C). The isolate - July, 1971, mud no. 3 - which had been identified as Aeromonas, had the same biochemical reactions as 27Gr 50, apart from being oxidase-positive. The flagella were not

examined. The third isolate - December, 1970, open water no. 25 - which had polar flagella was negative in the tests mentioned above except that gelatin liquefaction was not tested, and was designated Vibrio.

Arginine utilization by selected isolates

On the basis of the generic groupings made, both arginine-positive and arginine-negative isolates were found in each genus except for Escherichia (Table 5.5). The four Escherichia isolates were arginine-negative. The results for sixteen isolates of Enterobacteriaceae which were not identified to genus are not shown in Table 5.5. Seven were arginine-positive and nine were arginine-negative.

5.3 DISCUSSION

Identification of isolates as Enterobacteriaceae

Isolates were identified as Enterobacteriaceae using the scheme of Shewan, Hobbs and Hodgkiss (1960) and the general features of these bacteria are described on p.41. The majority of the isolates were very actively motile when young cultures were examined shortly after isolation. However, there were 21 isolates which fermented glucose rapidly and had a negative oxidase reaction but were non-motile. It is possible that these isolates were non-motile forms similar to the motile Enterobacteriaceae isolated but that motility was lacking when they were examined shortly after isolation. Carpenter, Lapage and Steel (1966) noted that Escherichia and Enterobacter were frequently non-motile if tested immediately after isolation.

TABLE 5.5 Arginine utilization by selected isolates

Grouped as	No. isolates giving reaction						
	Proteus	Salmonella	Escherichia	Enterobacter	Aeromonas	Vibrio	Total
Arginine-positive	4	2	0	9	2	0	17
Arginine-negative	2	4	3	4	0	1	14
Total	6	6	3	13	2	1	31[1]

[1] Of the group of isolates selected for further study, Mud, nos. 369 and 410; Water over weed, 3/70, no. 26 and Open water, 4/70, nos. 3, 12, 22, 38 were not tested.

The authenticity of 92 of the isolates on which additional tests were carried out was confirmed by their rapid fermentation of glucose in the medium of Hugh and Leifson and their negative oxidase reaction. Examination of the flagella of eight of the nine isolates giving atypical results for one of these two tests suggested that all but the three isolates, which were included for comparison, should still be grouped as Enterobacteriaceae as they had peritrichous flagella. The isolate whose flagella were not examined was included in the group selected for further study and the additional tests confirmed it was a member of the Enterobacteriaceae.

The tendency for isolates to have less vigorous growth on GYCA was probably related to the period, which was between one and two years, that the cultures had been maintained under mineral oil. The longer cells may have been caused by a slower rate of cell division. As rough variants were not found, it is unlikely that the biochemical behaviour of the isolates had changed.

Identification of isolates to genera of the Enterobacteriaceae

Only two of the isolates could ferment lactose with the formation of gas within 24 h. The majority of the 101 isolates tested were therefore not typical *Escherichia coli* strains.

The type species of many of the genera of the Enterobacteriaceae are of intestinal origin and are characterized by rapid utilization of test substrates at 37°C. Many of the biochemical tests differentiating the genera of the

Enterobacteriaceae are recorded after incubation for 24 h (Carpenter, Lapage and Steel, 1966). In attempting to identify the isolates from Lake Grasmere and its inlets which were selected for further study to described genera, there was the problem of how to interpret positive results which only occurred when isolates were incubated for longer than 24 h.

If isolates had shown results similar to described genera apart from, for example, slow lactose fermentation, then they could have been assigned to these genera fairly readily. However, in most cases the results of several tests did not allow the isolates to be identified using the key of Harrigan and McCance (1966) nor did they agree with any genera described by Skerman (1967). The two isolates which fermented lactose with the formation of gas within 24 h had MR and VP reactions typical of Escherichia but they did not utilize citrate.

Taylor (1941) found that certain isolates which could not ferment lactose at 37°C could do so at 30°C. In this study 35 isolates were incubated in McConkey broth at 30°C. This induced three isolates which had not previously fermented lactose to do so but these were only slight positive reactions and were not detected until after 4 days incubation (Table 5.3). Similarly, lowering the temperature of incubation had little effect on the speed with which lactose was fermented by most of the isolates. However, gas formation did appear to be enhanced at the lower temperature, but in no case was there an increase in gas formation or rate of lactose fermentation at 30°C such that the isolates gave results typical of coliforms if they had not done so at 37°C.

It is possible that the increase in numbers of isolates producing gas at 30°C was due to the different medium used for the 30°C incubation. These results are different from those of Taylor (1941) who found that half of his isolates which could not ferment lactose at 37°C could do so at 30°C. Taylor did not identify the cultures which fermented lactose at 30°C. They gave variable results to MR, VP and citrate utilization tests and no further differentiation could be made. This variability was characteristic of many of the isolates considered in the present study.

Isolates were also grown on Kliger's iron agar and incubated at 26°C. In some cases lactose fermentation was positive after 18 h where 48 h was required for lactose fermentation to occur at 37°C. Other isolates gave results which were difficult to interpret. Because of the complexity of the medium the results from Kliger's iron agar were not included as a comparable measure of lactose fermentation at 26°C.

Results from MR and VP tests were not clear in three cases even after repeating the tests with incubation for 4 days. Skerman (1967) recommends that if both MR and VP tests are positive after 4 days then a longer incubation period should be used to determine whether complete conversion of the acid will occur. This was not done with these isolates.

Salicin fermentation and urease production were both tested using tablets. These might not have detected positive reactions which, using media, could have been obtained only after prolonged incubation. As many of the isolates did not appear to have strong biochemical activity, standard

media would have been more suitable.

The lack of phenylalanine deaminase in all the isolates was unexpected especially as some were urease-positive and grouped as Proteus, which typically produces this enzyme (Skerman, 1967). As L-phenylalanine, which is the form commonly found in nature, was used in the medium either the isolates lacked this enzyme or possibly a longer incubation period than 24 h was required.

Production of ammonia from arginine proved to be of little value in differentiating between the genera of Enterobacteriaceae (Table 5.5). This confirmed the conclusion of Thornley (1960) that the arginine test cannot be used to distinguish Aeromonas from the Enterobacteriaceae and also suggested that a predominantly negative reaction by the Enterobacteriaceae as given in the key of Harrigan and McCance (1966) was misleading.

The results of the additional tests on selected isolates showed that, as suggested by the lactose fermentation data of all the isolates, none of the isolates tested further were typical strains of Escherichia coli. It is possible that some isolates might have become atypical after having been some period of time in water.

The value of assigning the isolates on which further tests were done to genera when they were not typical of the genera is questionable. However, the isolates did not form a homogeneous group and, as they could be divided into groups which had certain similarities with described genera, it seemed reasonable to consider them as strains of these genera. The isolates were grouped into five genera - Enterobacter,

Escherichia, Salmonella, Proteus and Serratia, of which Serratia contained a single isolate. Within the Enterobacteriaceae additional genera have been created for bacteria which are intermediate forms, e.g. Arizona and Citrobacter for types intermediate between Escherichia, Salmonella and Shigella. However, the statement of Stanier, Douderoff and Adelberg (1970) that "this generic hypertrophy really does nothing to help the problem of differentiation" is particularly relevant to this study. Most of the isolates were exceptions to either the five main genera given above or their intermediate forms and dividing the isolates into a larger number of genera would not have made ecological relationships any clearer.

Distribution of isolates in samples

There were some similarities between the Enterobacteriaceae isolated at the same time from different habitats. In November, 1969, samples from stream E and open water both included isolates which fermented lactose rapidly and were designated Escherichia. However, of the seven Enterobacteriaceae isolated from stream E, four were not able to ferment lactose. This indicated that they were not all similar to Escherichia and emphasized the need to do further tests on Enterobacteriaceae isolates to determine their relationship to coliforms. The source of the Escherichia-like isolates is most likely to have been stock as this stream does not pass near any human habitation. It is unlikely that the Escherichia found in the open water had originated from E

as this stream does not flow directly into the lake. However, as similar isolates were not found in any of the other samples analyzed it is likely that there was some common source possibly via another inlet.

The Enterobacteriaceae isolated from Elodea in October, 1969, were, except for one isolate, all unable to ferment lactose at 37°C. The three tested further gave very similar results and were distinct from the 32 other Enterobacteriaceae tested in their slight fermentation of lactose at 30°C but not at 37°C. The flagella of five isolates examined were subpolar on many of the cells. These isolates were not typical of the microflora found in the other weed samples analyzed (Table 6.1). It is possible that some snails may have been included in this sample and have been the source of these bacteria. The results of the tests suggested some relationship between the isolates and Salmonella.

In March and April, 1970, Enterobacteriaceae were widespread throughout the lake. As discussed in Chapter 4, the most likely source of these bacteria was the waterfowl. The two main groups which could have been associated with the birds were Enterobacter and Proteus but the forms of these genera found in the lake were atypical. Elodea was also sampled in March, 1970, and the single Enterobacteriaceae isolate was unlike those isolated from the weed in 1969. This suggested that the Enterobacteriaceae isolated in March did not form a part of the epiphytic microflora of the weed. The presence of any Enterobacteriaceae on the weed was

probably from coincidental lodging of bacteria from the water. In the water sampled in April and June, 1969, Enterobacteriaceae were not so common, but again most isolates were identified as Enterobacter.

The percentage of Enterobacteriaceae in the samples from over the harbour spring in March and April, 1970, was not as high as in the open water samples, but was higher than in the other sample from over spring H characterized (Table 5.1). Only a small proportion of the harbour Enterobacteriaceae were tested for lactose fermentation as many were non-motile (see p.145) so it cannot be concluded whether or not these Enterobacteriaceae were similar to Escherichia coli. However, as stock drink around the harbour it is possible that these isolates may have been typical coliforms.

The tests described in this chapter showed that few if any Escherichia coli were isolated from Lake Grasmere or its inlets. To some extent this was unexpected as stock are in close contact with the inlets and some of the lake shore. However, viability of coliforms in water is often limited. Gray (1951), in studying the microflora of a chalk stream, was unable to detect true Escherichia coli more than a few metres below the mouth of a tributary, which was contaminated by farm and domestic wastes.

Nevertheless, it is likely that although the Enterobacteriaceae isolated during this study could not be considered 'typical faecal coliforms', they were of faecal origin, having come from stock, waterfowl and possibly snails. As most samples in this study contained few Enterobacteriaceae isolates of any kind, although there were several sources around the lake, it is unlikely that these bacteria survive

for long in water and make any major contribution to the decomposition of organic matter in the lake.

This study has shown that there are many strains of Enterobacteriaceae which cannot be grouped satisfactorily into standard genera. Further work on these bacteria would determine whether they have any pathogenic qualities, in which case a means of identifying them more accurately would be important. At present, their main distinguishing features, as shown from the study of isolates from this lake and its inlets, are the variability of the reactions to standard tests given by the different strains and the difficulty in allocating them to described genera.

5.4 SUMMARY

1) Tests on a number of Enterobacteriaceae isolated from Lake Grasmere and its inlets failed to show that any were typical Escherichia coli. The majority fermented lactose only after 4 days at 37°C and, if they produced gas, it did not fill more than 10% of the inverted tube. They were identified as atypical forms of Enterobacter, Proteus, Salmonella, Escherichia and Serratia.

2) The isolates had probably originated from the faeces of animals, such as waterfowl, stock or snails.

CHAPTER 6

THE BACTERIAL POPULATION OF ELODEA CANADENSIS

As discussed in Chapter 3, the bacterial population of the water of Lake Grasmere is small compared to the thousands of bacteria per ml found in eutrophic lakes. However, there are extensive beds of Elodea canadensis (Fig. 2.1) in the lake, which it was considered might support a large bacterial population actively metabolizing nutrients on the plant surfaces. These bacteria might be attached directly to the plant surface or their normal habitat might be on the surface of other epiphytes, such as algae.

There has been little previous work on the microflora of aquatic plants. The epiphytic bacteria of Elodea canadensis were studied by Strzelczyk, Antczak and Kuchcińska (1971). They did not record the size of the population but studied the morphology, nutritional requirements, biochemical activity and antibiotic resistance of 60 isolates from each of water, mud and Elodea in a eutrophic lake. Further studies were carried out by Strzelczyk and Mielczarek (1971) on the ability of isolates from water, mud and Elodea to utilize a range of organic substrates.

Bacteria have been enumerated on Lemna minor (Coler and Gunner, 1969), Carex sp., Potamogeton sp. and a sedge (Potter, 1964), Sagittaria (Odum, 1957), and marine algae (Chan and McManus, 1969). The pigmentation of isolates was noted by Potter (1964) and Odum (1957). Chan and McManus (1969) characterized 25 isolates to genus.

The numbers of bacteria on other aquatic surfaces, such as stones, sand grains, pebbles and lake sediment particles, have also been studied (Potter, 1964; Anderson and Meadows, 1969; Batoosingh and Anthony, 1971; Tsernoglou and Anthony, 1971).

The phylloplane microflora of terrestrial plants has been studied more extensively and reviewed by Last and Deighton (1965). More recent studies have included those of Jensen (1971) on the bacteria on beech leaves, Klincāre, Krēslina and Mishke (1971) on the microorganisms of seven agricultural plants, and Stott (1971), who examined the microorganisms on green leaves of sugar beet.

The main difference between the microbial populations of aquatic and terrestrial plants is the absence of fungi on aquatic surfaces, and their significant contribution to the phylloplane microflora of terrestrial plants. In addition, large numbers of algal epiphytes occur on aquatic plants but they are not common on terrestrial leaves except in the tropics (Last and Deighton, 1965).

The extracellular release of dissolved organic matter from Najas flexilis and Scirpus acutus (Wetzel, 1969; Allen, 1971), amino acids from Lemna minor (Coler and Gunner, 1969), and phosphorus from Zostera marina L. (McRoy, Barsdate and Nebert, 1972) have been demonstrated. On the other hand, Bristow and Whitcombe (1971) did not observe any release of phosphate from shoots of Elodea densa although phosphate was absorbed by the roots. The shoots of Elodea spp. have been shown to assimilate both nitrate and phosphate in short term

studies (Wetzel, 1964; Bristow and Whitcombe, 1971).

Nutrients from the healthy photosynthesizing plant, from dead parts of the plant and from the lake water will be available to epiphytic bacteria. The concentrating 'surface effect' could also influence their activity. These bacteria may therefore be important in the recycling of nutrients in the lake, especially when the bacterial population of the water is small, and they may also be washed off the plant into the water to make up part of the lake population. In this chapter, the size and nature of the bacterial population on Elodea are examined and compared with the water population. The metabolic activity of the Elodea bacterial population is also considered.

6.1 METHODS

Open water and water over the Elodea canadensis beds were sampled from April, 1969, to July, 1971. Numbers of bacteria were estimated by the pour plate method and bacteria from selected samples were characterized. Details of sampling dates are given in Appendix 4.

Elodea was sampled on 13 occasions between October, 1969, and July, 1971. The numbers of bacteria were estimated by the plate method, using 5 g samples as described on p. 23, except in July, 1971, when the microflora of individual leaves was studied. In July, three categories of leaves were distinguished : young leaves within 1 to 3 cm of the shoot tip; mature green leaves; and brown, moribund but still entire, leaves further down the stem. The bacterial population of ten leaves in each of these categories

was estimated by the plate method and related to the surface area as described on p. 24. Bacteria from Elodea sampled in spring, late summer and winter were characterized. Details of sampling dates are given in Appendix 4.

Bacteria and epiphytic algae on Elodea leaves were counted directly in July, 1971, as described on pp. 54 and 55, respectively.

The viability of selected isolates of bacteria in lake water was studied so that an indication of the extent to which bacteria from Elodea were likely to make up a part of the water microflora could be obtained. Nine isolates from the lake were grown on nutrient agar for 5 days at 20°C. In Experiment 1 a small quantity of inoculum on a needle was transferred to 15 ml of filter-sterilized lake water. One hundred- and ten thousand-fold dilutions were plated in duplicate immediately and after 24 h incubation at 20°C. In Experiment 2, cells from 4-day-old nutrient agar slants were suspended in 15 ml of filter-sterilized lake water and incubated at 20°C. A loop of cell suspension was streaked on to a nutrient agar plate daily for 5 days and then at 1 to 4 day intervals for a total of 13 days.

As a test of the technique used to prepare the first dilution of Elodea, the effect of maceration on the viability of bacteria was examined. A small quantity of inoculum of a Cytophaga isolate cultured on a slant of Cytophaga medium (Anderson and Ordal, 1961) for 3 days, was transferred to filter-sterilized lake water. After 24 h the suspension was diluted with sterile distilled water to give a final con-

centration similar to the total number of viable bacteria in lake water. One hundred ml of this bacterial suspension was macerated for 4 min in the same way as described for the Elodea samples (p. 23). Samples were plated out on nutrient agar in quadruplicate before and after macerating.

The metabolic activity of the bacteria on Elodea leaves was studied by labelling leaves with ^{3}H-glucose or ^{3}H-thymidine and detecting uptake by autoradiography as described on pp. 81 and 82. Leaves of different ages were labelled in April, June and July, 1971. A single autoradiogram was prepared from each batch of 2 to 3 leaves. For each treatment, an autoradiogram from a control batch of leaves fixed before labelling was included. Autoradiograms from replicate batches of leaves from the same stem were set up for four of the treatments and three counts of the proportions of labelled and unlabelled cells were made on different areas of these autoradiograms. A single count was made of the other autoradiograms. The sample size for each count was determined as described on p. 66.

6.2 RESULTS

Seasonal fluctuations in the bacterial population of Elodea canadensis and lake water

The seasonal variation in numbers of bacteria on Elodea, in water over Elodea and open water is shown in Fig. 6.1. On the Elodea, a bacterial population between $2.55 \times 10^5 \pm 1.15 \times 10^5$ and $22.5 \times 10^5 \pm 11.0 \times 10^5$ per g fresh wt of Elodea was found throughout most of the year except for peaks in September and December, 1970, and a very large population of 2560×10^5

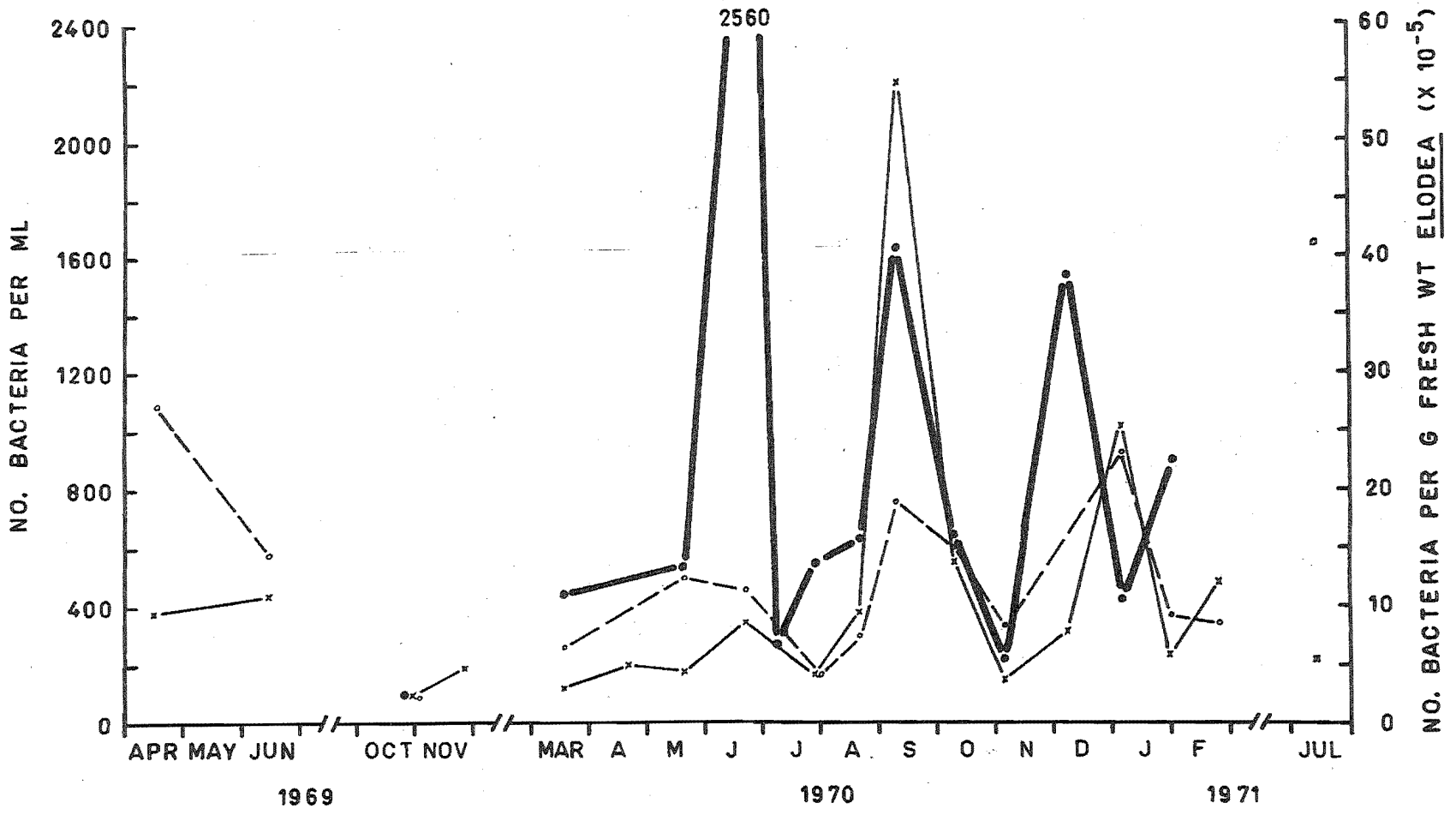


FIG. 6.1 Seasonal fluctuations in numbers of bacteria on Elodea canadensis and in lake water. Bacteria from open water ——×——, water over Elodea ——∘——, Elodea ——•——.

$\pm$ 320x10^5, recorded in June, 1970.

In September, 1970 large numbers of bacteria were also recorded in the open water but in the water over the Elodea, the bacterial population, although higher than in the previous month, was much smaller than the population of the open water. The large population on the Elodea in December, 1970, cannot be compared with the numbers of bacteria in the water over the Elodea as this water sample gave an abnormally high count of apparently a single species and the results for this sample were discounted. The open water population at this time was not high. The exceptionally high number of bacteria recorded on Elodea in June, 1970, was unexpected. The sample appeared healthy and, although the bacteria from this sample were not characterized, they were not obviously unusual.

The bacterial populations on Elodea growing in 2 and 6 m of water were examined in October, 1969. The size of the populations was very similar, as were the numbers of bacteria in the water sampled from over each of these weed beds, viz. Elodea:- 2 m - 2.55x10^5 $\pm$ 1.15x10^5; 6 m - 3.6x10^5 $\pm$ 0.6x10^5 (bacteria per g fresh wt); water over Elodea:- 2 m - 100 $\pm$ 50; 6 m - 115 $\pm$ 30 (bacteria per ml).

The higher numbers of bacteria in the water over the Elodea than in the open water in the autumn to winter months - April, 1969, May, June, 1970 and July, 1971 - may have been related to the presence of Elodea. These peaks in 1969 and 1970 were followed by slight increases in numbers of bacteria in the open water in June, 1969, and June, 1970 (Fig. 6.1).

Kinds of bacteria on Elodea canadensis and in lake water

Bacteria were characterized from Elodea, water over Elodea and open water in October, 1969, March, 1970 and July, 1971. In addition, bacteria from the water over Elodea and open water but not from Elodea itself were characterized in April and June, 1969 (Table 6.1).

The spring samples collected in October, 1969, included weed growing at two depths. In both these samples of weed, pseudomonads made up a major part of the bacterial flora, and flavobacteria and coryneforms were present in small numbers (Table 6.1). The only marked difference in the bacterial population of the two samples was that Enterobacteriaceae made up 23% of the bacteria characterized from the weed growing in 2 m of water, but were not found in the deeper weed sample. The water samples from over the two depths of weed had a similar range of bacteria to the samples of weed except that Enterobacteriaceae were not common, flavobacteria were present in smaller numbers, and there were more coryneforms in the shallower water sample than in the deeper water sample and the weed samples. The sample from the open water differed from all the other samples in the larger percentage of Gram-positive bacteria found (Table 6.1). However, only half as many bacteria were characterized from this sample and they may not have been fully representative of the water population at this time.

The samples collected in March, 1970, represented a late summer sample, when the water temperature was decreasing. The bacterial population of the weed sample was similar to the spring samples in the predominance of pseudomonads but

TABLE 6.1 Kinds of bacteria on Elodea canadensis and in lake water

Date sampled	Sample	No. characterized.	Percentage distribution											
			Alcaligenes/ Achromobacter	pseudomonads	flavobacteria	Cytophaga	Vibrio extorquens	Enterobacteriaceae	Aeromonas/ Vibrio	Acinetobacter	Chromobacterium	coryneforms	Micrococcaceae	Bacillus
15/ 4/69	Open water	96	6	8	33	12	27			+		7	+	+
15/ 4/69	Over Elodea	67	12	25	18	21	6	+				8	6	
17/ 6/69	Open water	74	+	15	13	22	8	9	+	+		12	10	+
17/ 6/69	Over Elodea	72	+	8	21	8	24	+				22	13	
28/10/69	Open water	23		43	+							13	22	17
28/10/69	Over Elodea growing in 2 m	46	+	61	+							29	+	
28/10/69	Elodea growing in 2 m	44	+	51	9			23				13		+
28/10/69	Over Elodea growing in 6 m	46	12	63	+			+				9	6	+
28/10/69	Elodea growing in 6 m	47	+	15	6							11	+	
17/ 3/70	Open water	51	+	23	+			51	20					
17/ 3/70	Over Elodea	47	11	32	+			34	11	+		+	+	+
17/ 3/70	Elodea	47	6	64	26			+				+		
13/ 7/71	Open water	44	+	28	22		8		+		+	33		+
13/ 7/71	Over Elodea	41	+	11	25						+	56	+	
13/ 7/71	Young leaves	40	+	26	53					+	7	7		+
13/ 7/71	Mature leaves	38	+	27	46				+	+		17		
13/ 7/71	Moribund leaves	44	+	37	46				9			+		

+ ≤ 5%

differed in the smaller number of coryneforms found. Also in March, flavobacteria were more common than they were in October. The bacteria of the two samples of water collected in March differed both from the sample of weed collected at this time and from all the samples taken in October, in the abundance of Enterobacteriaceae and Aeromonas/Vibrio (Table 6.1).

The winter samples were collected a year later in July, 1971. Samples of leaves of different ages were included. Considering the three samples of leaves as a whole, it can be seen that the bacterial flora of the Elodea at this time was different from the previous samples of Elodea (Table 6.1). Flavobacteria made up a larger part of the population and there were fewer pseudomonads. According to the broad grouping of bacteria in Table 6.1, the bacterial flora of the different aged leaves appeared to be similar. However, a further division of the flavobacteria on the basis of motility showed that there were more motile and fewer non-motile flavobacteria on the moribund leaves than on the healthy leaves (Fig.6.2).

In the July samples from the open water and water over weed, the same three groups - pseudomonads, flavobacteria and coryneforms - were found, but the numbers of bacteria in each group tended to be different from those in the samples of Elodea (Table 6.1.). Coryneforms were more common in the water samples and flavobacteria less abundant. Dividing the flavobacteria of the water samples into motile and non-motile groups indicated that non-motile flavobacteria were less common in the water than on the Elodea, and made up an even smaller proportion of the population in the open water.

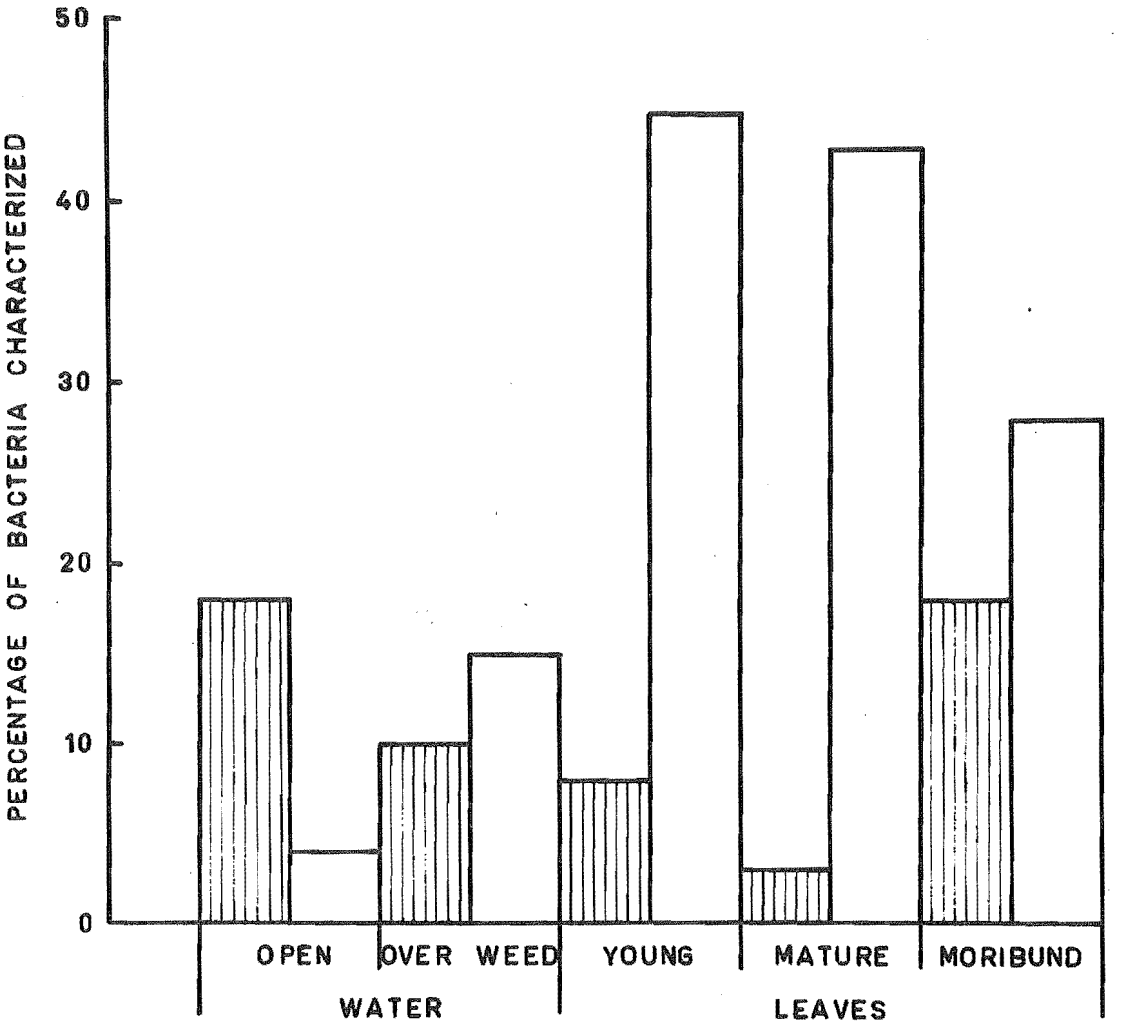


FIG. 6.2 Further division of flavobacteria isolated from samples collected in July, 1971

▥ - motile flavobacteria; ⊓ - non-motile flavobacteria.

Motile flavobacteria, on the other hand, were more abundant in the open water than in the water over Elodea and on the young and mature leaves, but the percentage in the open water was similar to that on the moribund leaves (Fig. 6.2).

Two additional sets of water samples were collected in April and June, 1969 although Elodea was not sampled at this time. In April, Vibrio extorquens was common in the open water and some isolates were found over the weed. In June, 1969, this bacterium was again present but higher numbers were found in the water over the weed than in the open water. Apart from this species, the main differences between the samples of water, collected in April and June, were that more coryneforms and Micrococcaceae were isolated in June. In the sample of water over Elodea collected in April, there were more Alcaligenes/Achromobacter, pseudomonads and Cytophaga, but fewer flavobacteria and Vibrio extorquens than in the open water sample. In June this trend was reversed with fewer pseudomonads and Cytophaga, but more flavobacteria and Vibrio extorquens in the sample of water over Elodea than in the open water sample. The proportions of Enterobacteriaceae and coryneforms in these two samples also differed.

Thus, there were seasonal differences in the proportions of the three main groups of bacteria - pseudomonads, flavobacteria and coryneforms - found in the samples of Elodea and lake water, and also in the abundance of groups, such as Enterobacteriaceae, Micrococcaceae, Vibrio extorquens and Cytophaga, which only sometimes made up a significant part of the microflora of these samples. As well as these seasonal

fluctuations, there appeared to be an association of some kinds of bacteria with a particular habitat.

In Table 6.2 the kinds of bacteria isolated from Elodea, water over Elodea and open water, when they were sampled at the same time, are summarized. Gram-negative bacteria were predominant in all three habitats with the largest percentage being isolated from Elodea. Chromogens made up a significant part of the isolates from the three sets of samples, but more pigmented bacteria were isolated from Elodea, sampled at these times, than from the water. Pseudomonads and flavobacteria were more commonly found in the samples of Elodea than in the water samples, but there were fewer 'Aeromonas/Vibrio and Enterobacteriaceae', coryneforms and cocci on the weed. There were some differences between the two water samples: pseudomonads and coryneforms were more common in the water over the weed, but flavobacteria and 'Aeromonas/Vibrio and Enterobacteriaceae' were not so numerous. A further separation of the bacteria grouped as flavobacteria showed that non-motile flavobacteria were more abundant in the weed samples than in the water samples, but more motile flavobacteria were isolated from the water than from the weed. Two of the motile isolates from the open water were similar to Xanthomonas, but none resembled Erwinia herbicola. Cytophaga was not found in any of the samples of Elodea characterized. No further separation of the other groups was attempted except that the number of fluorescent pseudomonads was recorded. These were found in all three habitats examined but did not make up a major part of the pseudomonads isolated from the weed or water over the weed.

TABLE 6.2 Kinds of bacteria isolated from lake water and Elodea canadensis in October, 1969, March, 1970, and July, 1971

Sample	Total number characterized	Percentage distribution					
		pseudomonads	flavobacteria	Aeromonas/ Vibrio and Enterobacteriaceae	coryneforms	cocci	others
Open water	118[1]	29	11	31	15	4	10
Water over Elodea	180[2]	42	7	13	23	4	11
Elodea	260[3]	48	30	7	8	1	6

Further division of pseudomonads and flavobacteria

Sample	Percentage distribution			
	pseudomonads		flavobacteria[4]	
	fluorescent	non-fluorescent	motile	non-motile
Open water	32	68	61	39
Water over Elodea	17	83	54	46
Elodea	10	90	19	81

[1] Of these bacteria 77% were Gram-negative and 30% were pigmented.

[2] Of these bacteria 73% were Gram-negative and 24% were pigmented.

[3] Of these bacteria 90% were Gram-negative and 37% were pigmented.

[4] Characteristics of these flavobacteria groups are described on p. 43.

They made up, however, a significant proportion of the pseudomonads of the open water.

Viability of lake isolates

a) in filter-sterilized lake water (Table 6.3)

Vibrio extorquens, Cytophaga and the flavobacterium isolate (non-motile) multiplied during the 24 h incubation period and were viable after at least 8 days. Pseudomonas fluorescens and the Enterobacteriaceae isolate decreased in numbers after 24 h, but some viable cells were found after 13 days. The coccus, coryneform and the two pseudomonad isolates did not increase in numbers in 24 h and were viable for only a short period of time.

b) after maceration

An isolate of Cytophaga was used for this experiment as this genus did not appear in any of the Elodea samples. The plate counts conformed to a Poisson distribution and after square root transformations were analyzed with a 't' test as described on p. 21. There was no significant difference between the means which were 169 bacteria per ml before macerating and 166 bacteria per ml after macerating.

Epiphytic microorganisms on individual leaves

Bacteria on individual leaves were studied in July, 1971. The plate counts and direct counts gave very different estimates of the sizes of the bacterial populations (Table 6.4). According to the plate count data, there were just over twice as many bacteria on the mature leaves as on the young leaves, but there was little increase in the bacterial

TABLE 6.3 Viability of selected lake bacteria in filter-sterilized lake water

Isolate	Experiment 1			Experiment 2
	No. bacteria (per ml x 10^{-4})		% viable after 24 h	viability (days)
	0 h	24 h		
Vibrio extorquens 14P 39	15.0 ± 4	90.0 ± 3	600	8 (+)
Cytophaga 13P 1	138.0 ± 3	265.0 ± 6	192	13 (+)
flavobacterium 6D3 33	95.0 ± 5	155.0 ± 21	163	13 (+)
Pseudomonas fluorescens 6D3 7	15.0 ± 1.4	3.4 ± 0.3	23	13 (+)
Micrococcaceae 3D 11	21.0 ± 4	4.7 ± 1.4	22	4
coryneform 6W 24	1.65 ± 0.08	0.07 ± 0.02	4	3
pseudomonad 6W 5	16.0 ± 3	0.1 ± 0.09	0.6	5
Enterobacteriaceae 6D3 3	141.0 ± 13	0.27 ± 0.08	0.2	13 (+)
pseudomonad 6E 9	8.0 ± 0	0.01 ± 0.005	0.1	5 (+) < 8

(+) viable on final day tested

population as the leaves died. The results from the direct counts however showed that the mature leaves had about 16 times as many bacteria on them as the young leaves and that there was a further four-fold increase in the population when the leaves died. The percentage of the total population which was cultured decreased from 26% to 1% as the leaves aged. Assuming the mean area covered by one bacterial cell was 1 μm^2 (Batoosingh and Anthony, 1971), the percentage cover of the leaves by bacteria was calculated (Table 6.4). According to the plate counts the maximum cover was 0.14%, while the direct counts indicated that over 14% of the moribund leaves were colonized by bacteria.

In July, 1971, the numbers of epiphytic algae of the genus Cocconeis on different parts of leaves were also counted. The mean area covered by one alga was 119 $\pm$ 21 μm^2 (p. 55) and using this figure the percentage cover of the leaves by algae was calculated for each microscope field (area, 0.14 mm^2)(Fig. 6.3, Table 6.4). Distribution was patchy on the young leaves, with parts of the leaves having no algae on them. The upper surfaces of the young leaves near the tips had the most algae on them. The mature and moribund leaves carried much larger populations. On the mature leaves, the algae tended to be evenly distributed over the upper and lower surfaces of the leaves. On some areas of the moribund leaves the largest numbers of algae were counted, but in other places, often on the upper surface of the leaf, algal numbers were smaller. The low numbers of algae at the base of many of the leaves may have been partly due to mechanical damage when the leaves were detached from the stem.

TABLE 6.4 Bacterial and algal populations on Elodea canadensis leaves of different ages

Age of leaf	Bacterial population					Algal population
	plate count		direct count		Mean % of bacterial population cultured	
	no. per mm^2 ($x10^{-3}$)	Mean % cover (per mm^2)	no. per mm^2 ($x10^{-3}$)	Mean % cover (per mm^2)		range of % cover (per 0.14 mm^2)
Young	0.595 ± 0.14	0.06	2.295 ± 1.09	0.23	26	0 - 14
Mature	1.375 ± 0.24	0.14	36.220 ±16.53	3.62	3.8	0 - 74
Moribund	1.450 ± 0.15	0.14	144.300 ±42.3	14.43	1.0	4 - 78

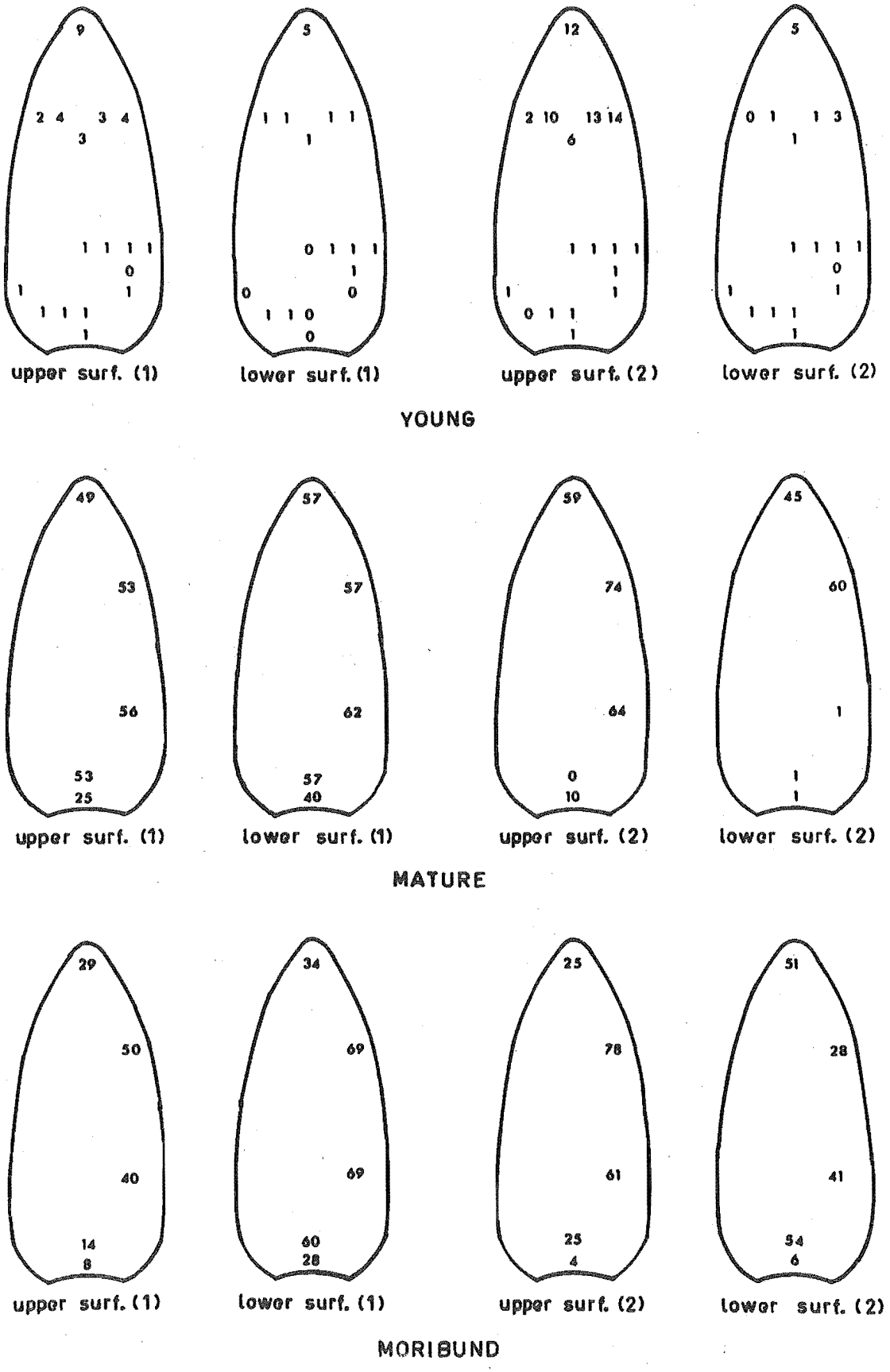


FIG. 6.3 Distribution of algae on Elodea canadensis leaves sampled in July, 1971. Percentage cover per microscope field (0.14 mm^2); scale: ⊢⊣ diameter of microscope field.

Metabolic activity of epiphytic bacteria

The metabolic activity of leaf bacteria *in situ* was investigated using an autoradiographic method. The three counts of labelled cells from each autoradiogram of mature and young leaves, labelled with ^{3}H-glucose in July, had very small standard deviations, viz. 18±2; 22±1; 19±2; 15±1.5 (% labelled cells). This had also been previously found with samples of moribund leaves collected in June (Table 2.9) and pure cultures (p. 67). Table 6.5 shows the percentage of labelled cells in the different treatments. For the treatments where autoradiograms were prepared from replicate batches of leaves and three counts were made per autoradiogram, the data shown in Table 6.5 are the average and standard deviation of the mean counts from the autoradiograms of the different batches.

No labelled bacteria were visible in any of the control autoradiograms of dead bacteria. The different aged leaves labelled in July were each from a sample, some of which was used to count bacteria and epiphytic algae. The labelling showed that the proportions of bacteria taking up ^{3}H-glucose and ^{3}H-Tdr on young and mature leaves were similar, but that there was a marked increase when the leaves died. These results can be compared with the proportions (shown in Table 6.4) of the bacterial populations on the different aged leaves, which were cultured. On the young leaves the percentage of the bacteria that was labelled with ^{3}H-glucose was more similar to the percentage that was cultured than on the mature or moribund leaves.

Although the percentage of metabolizing bacteria on the young and mature leaves was similar, the total number of metabolically-active bacteria on the mature leaves would have been far greater because of the larger bacterial population present (Table 6.4).

The possible effect of low temperatures on the activity of bacterial populations can be seen by comparing the percentages of labelled bacteria from moribund leaves sampled in June and July (Table 6.5). The water temperature in June was 7.6°C, which was nearly twice the July temperature. Approximately twice as many bacteria were labelled both with ^{3}H-glucose and ^{3}H-Tdr in June than July.

The April samples included mature and older mature leaves. The ages of the leaves were not directly comparable with the July samples but did show again that healthy leaves have a metabolically-active population on them (Table 6.5). A larger percentage of bacteria taking up ^{3}H-glucose would have been expected with the higher water temperature, but the percentage on the older leaves was comparatively low.

The percentage of bacteria taking up ^{3}H-Tdr was always less than the percentage of ^{3}H-glucose-labelled bacteria, but there was a tendency for the ratio ^{3}H-Tdr : ^{3}H-glucose counts to increase as the percentage of the population metabolizing ^{3}H-glucose increased.

TABLE 6.5 Metabolic activity of bacterial populations on Elodea canadensis leaves: uptake of ^{3}H-glucose and ^{3}H-thymidine

Date sampled (1971)	Age of leaf	Batch	Bacteria labelled (%) Glu[1]	Tdr[1]	Tdr/Glu	Water temperature (°C)
7 April	mature	I	32		0.59	14
		II		19		
7 April	older mature	I	17		0.47	14
		II		8		
9 June	moribund	I,II,III IV,V,VI,VII	82 ± 9	69 ± 6	0.84	7.6
13 July	young	I,II	17 ± 3		0.47	3.9
		III		8		
13 July	mature	I,II	20 ± 3		0.5	3.9
		III		10		
13 July	moribund	I	45		0.60	3.9
		II		27		

[1] Glu - ^{3}H-glucose; Tdr - ^{3}H-thymidine

6.3 DISCUSSION

Seasonal fluctuations in bacterial populations of Elodea canadensis and lake water

Bacteria on Elodea are influenced by several factors. They are subjected to the same changes in temperature, inflow of nutrients from outside the lake and organic matter derived from plankton blooms as affect the bacteria in lake water, but, in addition, the epiphytic bacteria are influenced by the plant and any other epiphytes which are competing for nutrients or space. Elodea plants consist of shoots of all ages and, in a 5 g sample, the number of bacteria will be affected by the proportions of healthy and moribund leaves.

With an increased rate of growth, as found in the spring months when the water temperature is rising, there will be a larger proportion of young shoots. As young leaves of plants are less susceptible to leaching (Tukey, 1971) a smaller supply of nutrients will be available and this, combined with the time required for colonization of a leaf to take place, will reduce the numbers of bacteria per g when Elodea is growing rapidly. As the leaf is also being colonized by algae there may be interaction between the bacteria and epiphytic algae. Conversely when the growth rate of Elodea slows down in autumn and winter, more bacteria per g would be expected with the larger proportion of older shoots and decomposing leaves.

The results from the regular sampling of Elodea (Fig. 6.1) only confirmed these hypotheses to some extent. The numbers of bacteria on Elodea in the autumn to winter months, between May and August, were not high except in June.

Although the weed did not cease growing during winter and some young shoots were always present in these samples, a population at a consistently higher level than in the spring and summer months was nevertheless expected. It is possible that the presence of moribund leaves in the sample reduced the numbers of bacteria obtained by the plate method. The results from the experiments with individual leaves (Tables 6.4 and 6.5) suggested this possibility which is discussed further on p. 201.

The population recorded on Elodea in June, 1970, was exceptionally large. The high count could have been due to the inclusion of fewer moribund leaves which, in other samples, were reducing the viability of many of the weed bacteria when the sample was macerated. This result may therefore have been more representative of the size of the population of the leaves in winter than the other counts.

The smaller bacterial population on the weed in November, 1970, can be correlated with an increased rate of weed growth as the water temperature rose, when bacterial colonization of the weed lagged behind the growth of new shoots. Similar low numbers of bacteria were found on the weed in late October, 1969.

Following the small bacterial population in November, 1970, there was a peak in December. Colonization of newly-formed shoots at a faster rate by bacteria than algae could result in such a sudden large population of bacteria on the weed. The largest percentage cover by Cocconeis of any part of a young leaf was 14%. Numbers of bacteria might then drop when a balance between the epiphytic bacteria and algae

was reached. A smaller bacterial population was recorded in January. Odum (1957) found that on glass slides, suspended in a stream at the level of Sagittaria blade tips, there was less than 25% cover by Cocconeis after 15 days, but after 1 month there was 95% cover.

The numbers of epiphytic bacteria thus appeared to be influenced by the rate of growth of Elodea. Environmental factors, such as the temperature and nutrient content of the lake, will be affecting this rate of growth and these factors may also have a direct effect on the epiphytic bacteria. Moreover, the numbers of bacteria in the water around the weed may influence the size of the epiphytic population. In September, 1970, the bacterial population on the weed increased at a time when the lake was silty throughout. This increase, as discussed in Chapter 4, appeared to be the result of settling of silt particles with attached bacteria on to the weed.

Nevertheless, provided numbers of bacteria in the lake were not dominated by an external factor, as in September, 1970, the rate of weed growth appeared to be an important factor controlling the size of the bacterial population of Elodea. This was further substantiated by the similar size of the populations of bacteria on weed growing at two depths of water, studied in October, 1969.

The weed, as well as being a major factor controlling the numbers of epiphytic bacteria, may also influence the numbers of bacteria in the water around the weed. Larger numbers of bacteria have been found in shallow bays than in open water (Stark and McCoy, 1938; Potter and Baker, 1956; Fondén, 1969a). One possible cause of this difference may

be the presence of vegetation in the bays when more organic matter will be available for bacterial decomposition (Stark and McCoy, 1938). On the other hand Fondén (1969a) considered that the inflow of nutrients and bacteria were primarily responsible for a higher bacterial population nearer to shore. A drop in the rate of weed growth and consequent increase in the proportion of moribund leaves in Lake Grasmere may have been the cause of the larger number of bacteria found over the weed compared to the open water in the autumn to winter months (Fig. 6.1). In the open water, the effect of the weed on the numbers of bacteria was not marked but the higher numbers of bacteria in June, in 1969 and 1970, than in the previous months could have been due to the addition of nutrients and bacteria from the weed. These higher bacterial populations are unlikely to have been caused by an inflow of nutrients and bacteria as neither the numbers of bacteria over the weed nor in the open water were correlated with rainfall (Table 4.1). At other times of the year the numbers of bacteria in the water over the weed were not substantially different from the open water population except in September, 1970, when as discussed on p. 130, more bacteria appeared to have sedimented from the water over the weed than from the open water.

From the study of the size of the bacterial populations on Elodea and in the lake water, near and away from the weed, it seemed that the state of growth of the weed was influencing the numbers of bacteria on the weed and, at times, the numbers of bacteria in the water.

Kinds of bacteria on Elodea canadensis and in lake water

The role of the weed in determining the kinds of bacteria found on the weed was examined by comparing the seasonal variation in kinds of bacteria on the weed and in the water. Interpretation of the data on kinds of bacteria on Elodea was complicated by the possibility that the range of kinds of bacteria growing on the plates might not have been representative of the heterotrophic bacterial population. It has been assumed that if any inhibition of bacterial growth occurred when the bacteria were plated out, it was not selective and the proportions of different kinds of bacteria were unaltered.

The main source of bacteria for colonization of new shoots is presumably the water around the shoots. These bacteria may have come from other leaves or from other parts of the lake. The ability of bacteria to survive in the water until a shoot is reached and their ability to colonize the new substrate in competition with other bacteria will both influence the composition of the bacterial flora on Elodea.

While there was a broad similarity in the main groups of bacteria found in the Elodea samples at different times of the year, the populations varied to some extent. Further, although on one occasion the populations from the water over weed and weed were very similar, at other times there were distinct differences.

In October, 1969, the kinds of bacteria on the weed growing at two depths and in the corresponding water samples were very similar, but, in March, 1970, the weed microflora was distinct from both the weed microflora of October in the small number of coryneforms and from the water populations

of March in the near absence of Aeromonas/Vibrio and Enterobacteriaceae, which were common in the water at this time (Table 6.1). The very small proportion of coryneforms may have been related to the low numbers of these bacteria in the water. Competition on the plant surface may have eliminated this group of bacteria from the weed and conditions in the water may not have been suitable for its survival and growth so introduction of these bacteria from another source did not occur. The poor representation of Aeromonas/Vibrio and Enterobacteriaceae on the weed in March, 1970, when they were common in the water suggested that these groups of bacteria were not suited for colonization of Elodea.

In July, 1971, all the major groups of bacteria found in the water samples were also present on the weed but the proportions of the different kinds of bacteria in the samples varied (Table 6.1). The samples of weed characterized in July, 1971, consisted of individual leaves of different ages and the influence of the host plant on the composition of the bacterial flora was apparent when these weed samples were compared. As the leaves matured there was an increase in coryneforms but this group was rare on the moribund leaves. While the proportions of flavobacteria remained at a similar level on the different aged leaves, the proportions of motile and non-motile forms changed (Fig. 6.2). On the young and mature leaves the level of non-motile flavobacteria was high but when the leaves died, the percentage of these bacteria dropped. The non-motile flavobacteria appeared to be typical epiphytes of healthy Elodea. On the other hand, motile flavobacteria were at low levels on the young and mature leaves but increased in numbers when the leaves died. Thus,

while the groups found on the leaves were similar and reflected the kinds of bacteria in the water, which were controlled by physical and chemical factors of the environment, the proportions of each group were influenced by the state of growth of the host.

This combination of effects by host and physical factors has been noted by Sinha (1971), who studied the fungi of four solanaceous plants and found that some fungi were present on the plant throughout the year but that others were limited to times of certain weather conditions. In addition, the maturity of the leaf and the individual host influenced the kinds of fungi found.

Between the population in the water over the weed and the open water, some differences could be attributed to the effect of the weed on the population in the water over the weed. There was a larger proportion of flavobacteria in July over the weed and the percentages of motile and non-motile flavobacteria were more similar to those of the weed flavobacteria than to the flavobacteria of the open water.

In general, at a particular time, the two lake water populations were similar but any differences, as seen in July, 1971 and also April and June, 1969, might well have been due to the influence of weed on the water nearer to the weed bed.

Thus, it seems that seasonal variations in the kinds of bacteria in the water around the weed are reflected in the microflora of the weed, but certain groups of bacteria are better able to grow on the weed surface and this selection varies with the time of year. The distribution of

kinds of bacteria as a percentage of the bacterial isolates from each habitat (Table 6.2) confirmed that pseudomonads and flavobacteria, and non-motile flavobacteria in particular, were the dominant epiphytic bacteria, while coryneforms and 'Aeromonas/Vibrio and Enterobacteriaceae' were less common on the weed than in the water. However, these figures are only a guide as certain bacteria were not found in the water when the weed samples were collected although they were common in the water at the other times of the year. Thus, the lack of Cytophaga on the weed may have been because these organisms were not present in the water and conditions were not suitable for their growth. However, as bacteria were characterized from weed samples in spring, late summer and winter, it seems likely that if Cytophaga was an epiphyte of Elodea it would have been detected in one of the samples. The lack of this organism in the weed samples cannot be attributed to the effect of maceration as there was no significant reduction in the numbers of viable Cytophaga following maceration.

The epiphytic bacteria of Elodea have not previously been studied extensively. Strzelczyk, Antczak and Kuchcińska (1971) isolated 60 epiphytic bacteria from one sample of Elodea and the percentage of Gram-negative bacteria (93%) was very similar to that obtained in this study (Table 6.2). They also reported that cocci were not found on the weed although they made up 8% of the water population.

The percentages of pigmented bacteria on Elodea and other aquatic macrophytes have been noted and are not consistent. Strzelczyk and Mielczarek (1971) studied the metabolic activity of 58 epiphytic bacteria from Elodea of which

20 were pigmented. Odum (1957) found the percentage of pigmented bacteria from Sagittaria blades sampled twice within a month from one part of a stream averaged 15 and 17%, but Potter (1964) recorded a wide range in the percentage of chromogens isolated from macrophytes in a pond. This study has shown that differences in the proportions of pigmented bacteria could be due to the different kinds of bacteria in the water and the ages of the plant parts studied. A predominance of pigmented bacteria has been noted on terrestrial phylloplanes (Stout, 1960; Last and Deighton, 1965; Jensen, 1971; Klincāre, Krēslina and Mishke, 1971). From the results of the present study and the reports in the literature, pigmented bacteria would appear to often comprise some of the aquatic microflora but they may not always be a major part.

On terrestrial plants, lactic acid bacteria (Jensen, 1971; Klincāre, Krēslina and Mishke, 1971; Nicholson, 1972) and other cocci (Stout, 1960) have been reported in the phylloplane, but these kinds of bacteria were rarely found on Elodea. Jensen (1971), who studied the bacterial flora of green beech leaves in early and late summer, found that fluorescent pseudomonads made up a significant part of the population, particularly in early summer. On Elodea, these bacteria were present in low numbers in most samples but never made up a major part of the population. Thus, there are differences, such as the relative abundance of lactic acid bacteria and fluorescent pseudomonads, in the bacterial populations of terrestrial and aquatic plants.

Viability of lake isolates

While bacteria on Elodea may initially have come from water, conversely, in water many of the bacteria may have come from the weed. Survival in each habitat is influenced by nutrient availability and in water the ability of bacteria to survive when nutrient levels are low is important. This ability was considered in an experiment when certain lake isolates were suspended in filter-sterilized lake water (Table 6.3).

With the exception of the coccus 3D 11, all the bacteria which had been isolated from the open water remained viable for the longest periods. The coryneform and pseudomonad 6W 5, from water over Elodea, and the pseudomonad 6E 9 from Elodea did not multiply in 24 h and did not remain viable for the duration of the experiment. Although glass was present where nutrients would be concentrated (Stark, Stadler and McCoy, 1938), in the absence of particulate matter in the water some bacteria were unable to utilize the dissolved nutrients. There are insufficient data from this experiment to say whether all isolates of a certain genus would have similar viabilities. However, such experiments could provide useful information on which organisms are indicators of contamination from a particular habitat, as coliforms are indicators of faecal pollution, and which, because of their long viability, may be present in places far removed from their original source. The latter group may be able to grow in lake water with little additional nutrients and thus make up a true water population. The importance in the lake of Cytophaga and Vibrio extorquens,

which both remained viable for a long period, is discussed in Chapters 10 and 11 respectively.

Epiphytic microorganisms on individual leaves

The study of individual leaves of different ages in July, 1971, showed that the bacterial populations of older leaves were larger than those on younger leaves but that this difference was not clear from the plate count data (Tables 6.4 and 6.6). The results of other studies on the numbers of bacteria on macrophyte and inanimate surfaces are also shown in Table 6.6.

The plate counts of bacteria on Elodea leaves were within the range of the counts recorded by Potter (1964) for Potamogeton and sedge but larger than the populations found in samples from Carex. The plate and direct counts of bacteria on Sagittaria blades were larger. This is to be expected as a Sagittaria blade is more substantial than an Elodea leaf and supports a large population of epiphytic organisms other than bacteria. These epiphytes would increase the surface area available for bacterial colonization. The ratio between the direct count and plate count of bacteria on Sagittaria was similar to the ratio between the direct and plate counts from mature leaves of Elodea.

The numbers of bacteria which have been recorded on inanimate objects are, in some cases, similar to the size of the populations found on young or mature Elodea leaves (Batoosingh and Anthony, 1971; Tsernoglou and Anthony, 1971). Anderson and Meadows (1969) recorded some larger populations on marine sand grains using the direct method, but their plate

TABLE 6.6 Numbers of bacteria found on aquatic surfaces

Surface	No. bacteria (per $mm^2 \times 10^{-3}$) viable count	direct count	Reference
Carex sp.	0.0481 - 0.102	NC[1]	Potter (1964)
Potamogeton sp.	0.038 - 1.96	NC	Potter (1964)
Sedge	0.06 - 3.31	NC	Potter (1964)
Sagittaria	7.5[2]	238[2]	Odum (1957)
Elodea young leaves	0.595	2.295	present study
Elodea mature leaves	1.375	36.22	present study
Elodea moribund leaves	1.450	144.3	present study
Lake stones	0.13 - 18.1	NC	Potter (1964)
Lake sediment	NC	3 - 15	Tsernoglou and Anthony (1971)
Marine pebbles	3.45	23.8	Batoosingh and Anthony (1971)
Marine sand	0.0002 - 0.04	25 - 259	Anderson and Meadows (1969)

[1] NC - no count

[2] number per mm^2 calculated from data in Table 9 (Odum, 1957).

counts were much lower than those of Elodea bacteria. In general, the low level of bacterial colonization of young and mature Elodea leaves is similar to that of lake sediment and other such inanimate particles. This suggests that during the early stages of colonization there is little difference between leaves and inanimate objects. In both cases, some bacterial attachment is encouraged because nutrients are adsorbed to the surface from the surrounding water, but it is not until the leaf matures and later becomes moribund that there is an increase in the bacterial population of the leaf beyond that of an inanimate surface. The larger populations recorded on stones compared to those on plants, by Potter (1964) were probably related to the presence of many other living organisms on the stones which tended to promote bacterial colonization.

Potter (1964) noted that the counts of bacteria and the percentage of chromogens on plant surfaces varied over a wide range. She concluded that plants were not selective or possibly that the 1 cm^2 area sampled was not representative of the population. Numbers and kinds of bacteria on an aquatic surface will be influenced by such factors as the quantity of nutrients supplied by the surface, which may vary with age, and the nutrients in the surrounding water. Therefore, variability in counts and kinds of bacteria may not necessarily be an indication of a lack of selectivity. The data presented in this chapter, which show that differences were sometimes found between kinds of bacteria in water and on weed sampled at the same time (Table 6.1), that certain kinds of bacteria were more often isolated from weed than water (Table 6.2), and that the bacterial

population of the leaf increased as the leaf aged (Table 6.4), suggest that the weed surface is a specialized habitat.

The very much larger estimate of the bacterial population on the moribund leaves obtained by the direct microscopic method compared to the plate method suggested that, as the leaves aged, either

(i) the amount of some compound which inhibited bacterial growth on the pour plates increased; or

(ii) there was a larger proportion of dead bacteria; or (iii) certain bacteria were present, which could not grow on the medium provided, or

(iv) were killed during maceration.

The possibility that there might be some inhibitory compound present in older leaves was considered. This compound might be associated with the epiphytic algal population or with the leaf itself. Epiphytic algae covered the mature leaves and many parts of the moribund leaves densely. In terms of percentage cover, far more of the leaves were colonized by algae than bacteria. However, bacteria were not counted *in situ*. As it is likely that they were not evenly distributed there may have been some regions with greater percentage cover by bacteria which were comparable to the areas of dense algal cover.

Microscopic examination showed that very few of the *Cocconeis* had bacteria attached to them. This could have been the result of antagonism. However, the counts of algae on mature and moribund leaves were not markedly different. Any inhibition could not therefore be attributed to an increase in the numbers of algae as the leaves became moribund. It

is possible that if the proportion of dying algae was higher on moribund leaves, products released from senescent algae might have influenced the plate counts.

Major biochemical changes will also occur within the leaf as it ages. A declining rate of respiration is a characteristic of the leaf approaching senescence (Woolhouse, 1967; Baddeley, 1971). Baddeley studied the effect of extracts of different aged leaves of Ricinus communis on mitochondrial oxidations. She found that there was increased inhibition of these oxidations by the senescing leaves which was correlated with an increase in fatty acids in the leaves. Baddeley considered that the fatty acids might be partially responsible for the greater inhibition of mitochondrial oxidations. If similar compounds were present in ageing Elodea leaves or algae they might be released when the shoots were macerated and inhibit many bacteria and, thus, be one factor causing the increasing discrepancies between direct and plate counts. Unfortunately this possibility was not investigated.

Metabolic activity of epiphytic bacteria

The rationale of the method used to determine the percentages of metabolically-active bacteria on Elodea leaves has been discussed on pp. 62 and 68. Interpretation of counts of labelled cells from autoradiograms is not easy. The problem of lack of uptake of the labelled compound by bacteria which are, however, metabolizing other compounds, was discussed on pp.70-76. The possibility that bacteria might have accumulated the label passively although they were

not metabolizing, was excluded as none of the control autoradiograms showed any labelling. The variability in the bacterial populations between different batches of leaves has been demonstrated (Tables 2.9 and 6.5). There appeared to be greater variability between different batches of moribund leaves than among batches of young and mature leaves, but too few batches were examined for any definite conclusions to be drawn. However, the counts emphasized the need for replicate batches in experiments where the percentages of labelled cells in different treatments were likely to be close. Within an autoradiogram the variability was so small that the accuracy of a single count was well within the limit of error set by the sample sizes chosen.

The results of labelling the different aged leaves, sampled in July, 1971, with ^{3}H-glucose (Table 6.5) were interesting as they showed that the increasing discrepancy observed between the plate and direct counts as the leaf aged could not be attributed to a larger proportion of dead bacteria. Although the plate count of bacteria on moribund leaves was only a small fraction of the direct count, nearly half the bacteria on the leaves were alive and metabolizing at the time of sampling as they became labelled with ^{3}H-glucose. Either the method of obtaining the plate count was not suitable or, as discussed earlier, there was some inhibitor present in increasing quantities in the older leaves.

The effect of maceration on viability of bacteria was tested on only one isolate but no adverse effect could be detected. The possibility that the medium was less suitable for bacteria from moribund leaves was not investigated. However, it seems unlikely that a discrepancy which increases

with the age of the leaf could be caused by the method used to enumerate the bacteria which was the same for all the leaf samples. The possibility remaining is that there is an inhibitor associated with the moribund leaves. The autoradiograms showed that such an inhibitor could only be confined inside the leaf as many bacteria were able to metabolize on the surface of the leaf. It is unlikely that an inhibitor is present within the diatom cells as they would not be broken up on macerating. On the young leaves, the percentage of bacteria taking up ^{3}H-glucose and the proportion of the total population estimated by the plate method were similar, suggesting that an inhibitor was not present in the young leaves. In this case the difference between the direct count and plate count was probably caused by the presence of dead bacteria, autotrophic bacteria, or bacteria metabolizing very slowly which did not grow on the medium provided. Some of the bacteria counted directly on the mature and moribund leaves would also fall in to these categories.

Many bacteria lodging on the surface of leaves may remain there viable but not actively growing until conditions of nutrient supply or temperature are suitable. The autoradiographic experiments showed that the proportions of metabolically-active bacteria were higher when the leaves were moribund and when the water temperatures were higher. The data from the July experiment showed little difference in the percentage of metabolizing bacteria on young and mature leaves (Table 6.5). However, when the total number of bacteria on the leaves was taken into account (Table 6.4) it was seen that there were far more bacteria metabolizing on the mature

leaves. These results indicated that in a quantitative study the absolute number of metabolizing bacteria on a leaf should be determined as well as the proportion of the population which is active. Thus, while the older leaves sampled in April had a lower percentage of active bacteria on them than the younger leaves sampled at the same time, the older leaves could have had a larger active population on them.

The results from the labelling with ^{3}H-Tdr suggested that with increasing age of leaf, more of the metabolizing bacteria were dividing and, thus, growing more rapidly.

Ecological significance of epiphytic bacteria

The experiments examining the bacterial population of Elodea, which have been discussed in this chapter, showed that Elodea supported an epiphytic population of bacteria which increased in size and metabolic activity as the shoot aged, although initially the size of the population was comparable with that found on some inanimate objects, such as lake sediment particles. The leaf appeared to be a selective environment as the kinds of bacteria on Elodea did not reflect the population in the water exactly. This selectivity was probably caused by the release of nutrients from the leaf, which according to the data on the number of metabolically-active bacteria on the leaf surface, appeared to increase as the leaf aged.

The main nutrients required by Elodea are minerals. These may be provided from external sources and be assimilated from the water by the shoots (Wetzel, 1964; Bristow and Whitcombe, 1971). Alternatively, they may have been

sedimented on to the mud and be absorbed by the roots, often only after breakdown to assimilable compounds by bacteria. There is some controversy about the role of roots of Elodea in the uptake of minerals, but recent work by Bristow and Whitcombe (1971) on the uptake of phosphate by aquatic plants, has shown that, in Elodea densa, phosphate is taken up by the roots and translocated to the shoots. The roots of Elodea canadensis may play a similar role. A third source of minerals may be from bacterial recycling of cellular material of Elodea. Moribund shoots will be a major supply of such decomposable matter but compounds available for decomposition may be released extracellularly from healthy shoots, as suggested by the numbers of metabolically-active bacteria on mature Elodea leaves.

In Lake Grasmere the numbers of bacteria in the water over the weed were rarely very large or much greater than the population in the open water. This suggested that decomposition on the leaves was not resulting in any significant release of nutrients into the water which could be decomposed further. It is likely that the ratio between carbon and nutrients essential for bacterial growth on the leaf surface is only just sufficient to maintain bacterial activity and few nutrients are released from the bacteria. Any minerals which are released may be rapidly assimilated by the epiphytic algae and possibly Elodea itself.

Elodea may be able to compete more successfully than bacteria over a long period for minerals, by storing them in its tissues when the concentration in the lake water is higher and utilizing these minerals later when concentrations are limiting. Boyd (1969) found that the maximum rate of

absorption of mobile mineral nutrients occurred before the time of maximum growth of Justicia americana and Alternanthera philoxeroides - two emergent angiosperms. However, McRoy, Barsdate and Nebert (1972) considered that a continual supply of phosphorus was necessary to maintain Zostera marina L. They demonstrated active transport of phosphorus from sediment, through the plant to the surrounding water. On the other hand, Bristow and Whitcombe (1971) did not observe any excretion of phosphate into the medium by Elodea densa. In an environment where bacterial activity may be limited by the availability of certain minerals or trace elements, epiphytic bacteria may be essential in ensuring a rapid recycling of nutrients required by Elodea and epiphytic algae. Nutrients tend to be concentrated on to surfaces and, as epiphytes, these bacteria will be in a more favourable position to obtain any minerals required from the lake water than planktonic bacteria. Furthermore, any decomposition occurring on the leaf surface before the leaf falls off the plant and sediments on to the mud will be more efficient, as oxygen will not be limiting as it may be in the mud. Thus, epiphytic bacteria would appear to be of particular importance in the recycling of nutrients when bacterial populations in lake water are low.

6.4 SUMMARY

1) Bacteria on leaves of different ages were enumerated by direct and indirect methods. There were 63 times as many bacteria on the moribund leaves as on the young leaves according to the direct count but only just over twice as many according to the plate count. Labelling batches of leaves from the same samples with ^{3}H-glucose and ^{3}H-thymidine showed that the increasing discrepancy between the plate count and direct count estimates as the leaves aged was not due to a larger proportion of dead bacteria. It is suggested than an inhibitor in moribund leaves may be released when Elodea samples are macerated and thus reduce the viable count of bacteria.

2) The mean numbers of viable bacteria per g fresh wt of Elodea varied seasonally from 2.55×10^{5} to 22.5×10^{5}, with an exceptionally high count of 25.6×10^{7} recorded on one occasion. The possibility, noted in 1) above, that numbers of bacteria, in some cases, may have been reduced because the samples were macerated must be taken into account.

3) The kinds of bacteria on Elodea appeared to be influenced both by the environmental conditions and by the plant. There were some similarities between the population on the weed and in the water at different times of the year but certain groups, such as non-fluorescent pseudomonads and non-motile flavobacteria were more commonly found on the weed than in the water.

A comparison of the bacterial flora of different-aged leaves showed that non-motile flavobacteria were more common on the young and mature leaves than on the moribund leaves, but the opposite was true for the abundance of motile flavobacteria, with larger numbers on the moribund leaves and, also, in the open water, than on the healthy leaves.

4) The autoradiographic experiments carried out on the leaves of different ages showed that between 17% and 82% of the bacterial population might be metabolizing *in situ*. Numbers of metabolizing bacteria on the leaves increased considerably as they aged and died.

5) The proportion of bacteria from young *Elodea* leaves, labelled with ^{3}H-glucose, was similar to the proportion of the total population estimated by the pour plate method.

6) Larger numbers of bacteria were found in the water over the weed than in the open water in the autumn to winter months but this difference was not apparent at other times of the year.

7) The importance of epiphytic bacteria on *Elodea* leaves in the recycling of nutrients from *Elodea* is discussed.

CHAPTER 7

THE GENERAL RELATIONSHIP BETWEEN PLANKTON AND BACTERIA IN THE OPEN WATER

The main source of organic matter for bacteria in the open water of Lake Grasmere during most of the year was plankton. Aquatic birds added to the organic matter but compared to the amount of plankton in the water this increase was unlikely to have been significant, except possibly during the autumn to winter months when large numbers of birds came to the lake. Crops, tussock grasses and, to a lesser extent, forest were all possible external sources of detritus in the lake. There was, however, only one small inflowing stream which would carry detritus into the lake. Some particles would also have been blown into the lake but it is probable that detritus from external sources made up only a small proportion of the organic matter of the lake except after high winds or heavy rain. As plankton made up a predominant part of the organic matter of the open water, the response of bacteria to changes in the plankton populations was of particular interest.

This organic matter was represented by both phytoplankton and zooplankton at various stages of development. In addition to being a supply of organic matter when dead, phytoplankton may influence bacteria by releasing products of photosynthesis and respiration (Fogg, 1971), and zooplankton may graze either directly on bacteria or on their potential food (Fondén, 1969a) and also excrete nutrients

(Johannes, 1968).

Seasonal studies have shown that relationships between phytoplankton and bacteria may be extremely diverse. In a study of eight lakes, Potaenko and Mikheeva (1969) found that bacterial and phytoplankton populations could have maxima at the same time, be inversely related or vary independently of each other. The results of other authors further emphasize this diversity. Thus, Overbeck and Babenzien (1964) and Silvey and Roach (1964) found bacterial populations increased at the same time as, or even before, an increase in algal numbers; Henrici (1938), Schegg (1968) and Fondén (1969a) observed that bacteria responded to phytoplankton maxima only after a lag period. Taylor (1949) and Collins (1957) observed that although numbers of bacteria increased after large populations of blue-green algae had died, there was no significant increase following the death of a large crop of the diatom, Asterionella. Other authors have not been able to show any direct relationship between seasonal variations in numbers of phytoplankton and bacteria (Gerletti and Melchiorri-Santolini, 1968; Goldman et al., 1968; Sieburth, 1968; Štěpánek, 1968; Jones, 1971). The study of Goldman et al. also emphasized that a positive correlation between phytoplankton and bacteria could be misleading as both phytoplankton and bacteria could be correlated with a third variable, and not be dependent on each other.

Positive correlations have been found between zooplankton and bacteria (Goldman et al., 1968), but the relationship may vary. Fondén (1969a) found that there was a decrease in numbers of bacteria when filter-feeding

zooplankton populations increased. This was attributed to grazing of bacteria by zooplankton. The importance of bacteria in the diet of zooplankton has been considered by several workers including Nauwerck (1963) and Drabkova (1965). Drabkova studied the generation time and destruction of bacteria by zooplankton in samples of filtered and unfiltered lake water and concluded that, while in spring bacteria were the main food supply, at other times of the year phytoplankton and detritus were the major sources of nutrients. Thus, the diet of zooplankton in a lake will influence the relationship between zooplankton and bacteria.

The association of specific kinds of bacteria with phytoplankton and zooplankton has been examined in the marine environment. Sieburth (1968) observed that dominance of Flavobacterium was associated with blooms of a marine diatom, whereas Vibrio was apparently suppressed by the presence of large numbers of the diatom. However, in a study of the bacterial population of sea water and concentrated plankton of inshore waters of Japan, Simidu, Ashino and Kaneko (1971) found that Vibrio was the major genus in the plankton samples. The amount of plankton, especially phytoplankton, in the sea water was initially small. Large bacterial populations have been recorded in samples of concentrated plankton (Waksman et al., 1933; Rigomier, 1967; Seki, 1967; Simidu, Ashino and Kaneko, 1971).

Thus, previous work on the relationship between bacteria and plankton has shown that there are several possible responses of bacteria to changes in phytoplankton and zooplankton populations. In this chapter seasonal fluctuations in plankton and bacterial populations are examined and the

bacterial response to fluctuations in plankton populations is compared with those reported by other authors. The possibility of a bacterial flora specifically associated with the plankton is also considered.

7.1 METHODS

The numbers of algal cells were determined in 15 samples of open water between November, 1970, and July, 1971. Dimensions of the four most common algae were recorded, when they were dominant. Ten cells were usually measured and where algae were grouped in colonies or filaments, cells from different colonies or filaments were measured. The surface area and volume of the cells were calculated assuming the shape of Diatoma and Asterionella was a cuboid and that of Cyclotella and Melosira was a cylinder. The mean width of each Asterionella cell in girdle view was used to determine the width which was used in the calculations of surface area and volume. The breadth of the Cyclotella cells was determined from one sample in which the algae were so concentrated that a number of Cyclotella cells were standing on their sides. The mean breadth from this sample was used in calculations of volume and surface area for another five samples in which only the diameter of the cells was measured. The number of cells in unbroken filaments of Melosira was recorded and the surface area of Melosira was determined per filament.

Numbers of zooplankton were determined between September, 1970, and July, 1971. The author counted four

samples in November and December, 1970. The remaining data were obtained from Dr V.M. Stout.

Bacterial populations were estimated by the pour plate method, and samples collected between September, 1970, and July, 1971, are discussed in this chapter. Details of sampling dates are given in Appendix 5. Bacteria from samples of open water and plankton were characterized on four occasions.

Eleven experiments were carried out to determine whether part of the bacterial flora was associated with the plankton. Plankton was separated by filtering through a Millipore filter (pore-size, 3 μm), or concentrated by passing lake water through a net (mesh-size, 94 μm) or by centrifuging as described on p. 24. Numbers of bacteria in the various fractions were estimated by the pour plate method.

The bacterial flora of algae and individual members of the zooplankton was examined by placing organisms on nutrient agar and incubating at 20^{o}C for up to two weeks. Animals were isolated from vertical net hauls and the microflora of washed and unwashed organisms compared. They were washed in a bijou bottle of sterile water. This procedure was repeated once or twice with fresh bottles of sterile water. Individual algae were not isolated. The algal samples consisted of drops from a vertical haul which were examined to ensure no animal was included.

7.2 RESULTS

Estimation of quantity of plankton and bacteria

The numbers of algal and bacterial cells in the open water were compared. The size range of the two populations was very different, viz. algae - 1 235 to 10 910 cells per ml; bacteria - 55 to 1 020 cells per ml. Thus algae always outnumbered bacteria. The ratio of number of algae to number of bacteria from any one sample varied from 4:1 to 34:1.

The dominant algae were Diatoma elongatum (Lyngb.) Agardh, Melosira granulata (Ehr.) Ralfs var. angustissima Müll., Asterionella formosa Hass. and Cyclotella kützingiana Thwaites. The range of cell volume and surface area for the different algae over seven months is shown in Table 7.1. These figures were calculated from the measurements given in Appendix 6. Because Cyclotella kützingiana cells are small, counts of algal cells over-emphasized any peak in the algal biomass if Cyclotella was present in large numbers. In Fig. 7.1 the total surface area, volume and numbers of the dominant algae have been plotted. The large difference between numbers of algae and either surface area or volume at the beginning of February was due to the presence of many Cyclotella. As the algal populations were being considered as a source of organic matter for bacteria, the cell volume was a more useful parameter than cell number, and it was used when comparing changes in algal and bacterial populations.

For all the samples, following the fluctuations in volume of the four major algal species provided a good

TABLE 7.1 Range of volume and surface area of the dominant algae between November, 1970, and May, 1971.

Alga	Cell volume (μm^3)	Cell surface area (μm^2)	Mean volume / Mean surface area
Diatoma elongatum	497 - 571	875 - 932	0.58
Asterionella formosa	331 - 451	543 - 654	0.66
Melosira granulata var. angustissima	382 - 515	341 - 451[1]	1.11
Cyclotella kützingiana	44 - 95	71 - 127	0.69

[1] Mean surface area per cell calculated from filament surface area.

indication of changes in volume of the total phytoplankton. The remainder of the algal population made up less than 10%, by numbers, of the population in 10 of the 15 samples counted, between 13% and 18% in four samples and 48% in one sample collected in May, 1971. The numbers of all algae in the samples are given in Appendix 7. In May, 1971, the total number of algal cells was low and the alga which made up almost half the population was a small green unidentified flagellate, whose volume would have been small in comparison with that of the major diatoms present both at this time and in other samples.

The changes in the volume of each of the four algae

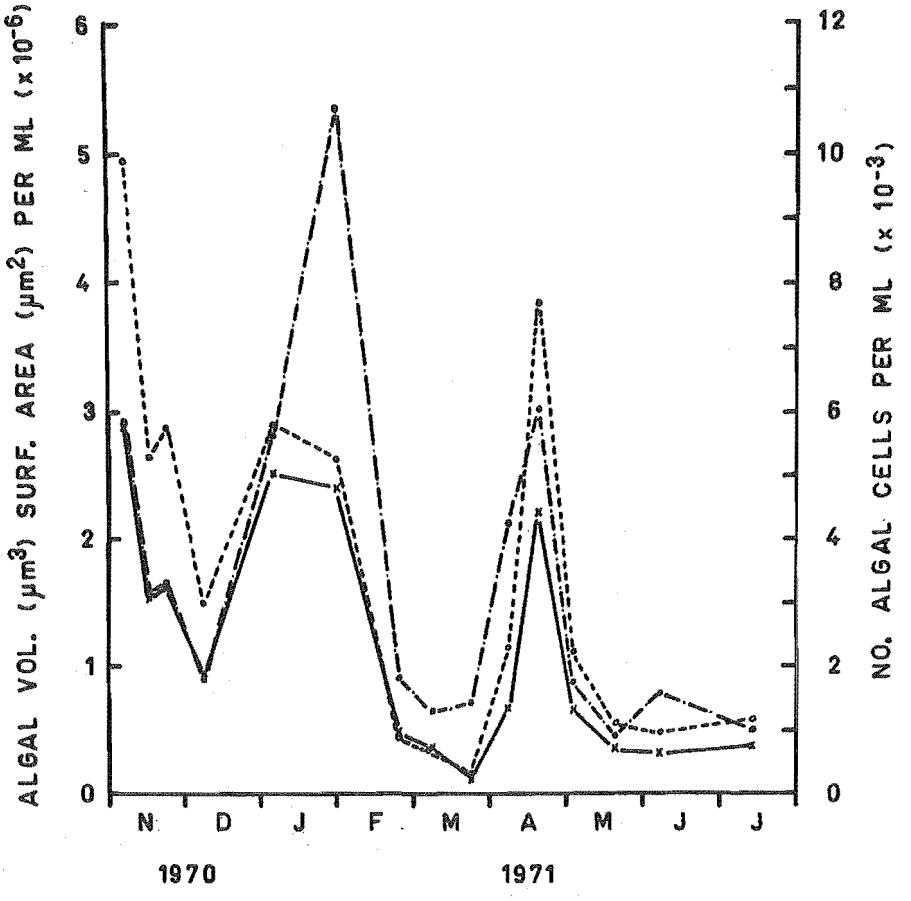


FIG. 7.1 Relationship between total cell number, surface area and volume of dominant algae. Number —·—•—·— ; surface area ---•--- ; volume —×— .

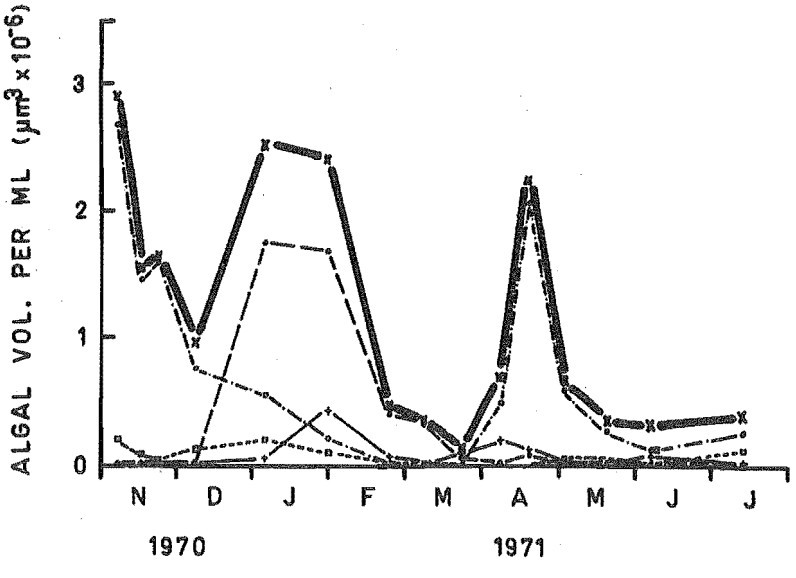


FIG. 7.2 Seasonal variation in total cell volume of dominant algae and cell volume of each alga. Total cell volume —×— ; volume of Diatoma —·—o—·— , Melosira —·—•—·— , Asterionella ----▫---- , Cyclotella —·—+—·— .

measured were compared with changes in total volume of all four algae (Fig. 7.2). As the peaks consisted of either almost entirely one alga, or several algae whose volume declined at the same time, summing the volumes of the individual algae did not mask any major fluctuations. Therefore in Fig. 7.3, the seasonal variations in total volume of the four dominant algae are compared with the numbers of bacteria and zooplankton.

The zooplankton consisted of Cladocera - Ceriodaphnia dubia and Bosmina meridionalis; Rotifera, of which the species most commonly found were Filinia longiseta, Keratella cochlearis, Polyarthra vulgaris, Synchaeta pectinata, Ascomorpha sp. and Asplanchna priodonta; Copepoda - Eucyclops sp. and a mite - Piona sp.. In the open water, adult cyclopoids were rarely found and it was, thus, unlikely that many of these animals were dying in the open water and providing a source of nutrients for bacteria. Mites were not found in large numbers. The changes in the populations of copepods and mites were, therefore, excluded from Fig. 7.3. The numbers of rotifers and Cladocera are shown separately because of the difference in their sizes.

Seasonal variations in plankton and bacterial populations

From September, 1970, to July, 1971, the effects of three algal maxima, and seven zooplankton peaks of various sizes, on the bacterial population were studied (Fig. 7.3).

The first algal peak, of which only the end of the bloom was included in this study, consisted mainly of Diatoma. From November to December, there was a drop in

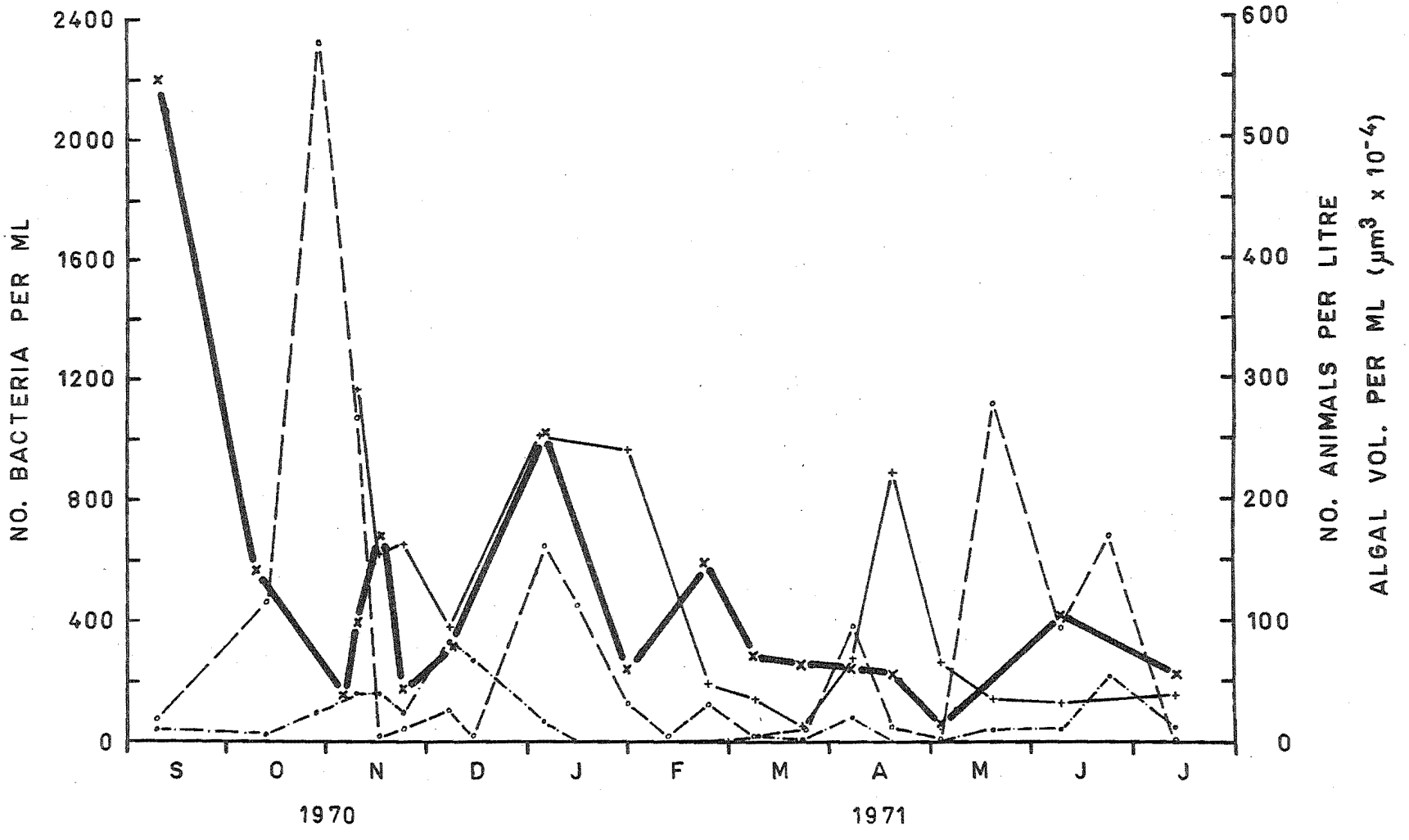


FIG. 7.3 Seasonal fluctuations in plankton and bacterial populations in the open water.
Bacteria —×—; diatoms —+—; rotifers — —∘— —; Cladocera —·—·— .

this algal population. The decline was monitored by three samples for plankton and bacterial counts taken at weekly intervals during November, and an additional sample for a bacterial count collected at the beginning of November. The bacterial counts showed that, although there was an increase in the numbers of bacteria between 5 and 16 November, when the algal population was decreasing, by 23 November, bacterial numbers had decreased although the algal volume was very similar on 16 and 23 November. When the algal volume decreased further between 23 November and 7 December, the bacterial population increased only slightly. In January and February, 1971, there was a summer peak of algae, made up of Melosira and Cyclotella, and, to a lesser extent, Asterionella. The beginning of the summer algal peak coincided with a marked increase in the numbers of bacteria but the bacterial population dropped while the numbers of algae were still at their maximum. A small increase in numbers of bacteria was recorded in late February. The third peak in the algal population was in April, 1971, and consisted of Diatoma and some Cyclotella. No marked bacterial response to either the increase or decrease in volume of algae was detected in April or May, 1971.

Some relationship between zooplankton and bacterial populations is apparent from Fig. 7.3. When the change in numbers of rotifers between 9 and 16 November was studied, it was seen that the peak in numbers of bacteria appeared to be more closely related to the sudden drop in the rotifer population during this period, than to the slower drop in the algal population. Numbers of bacteria also increased as rotifer populations decreased in February and June, 1971,

and bacteria were abundant at the beginning of January following a peak in Cladocera in December. Although increases in the zooplankton population sometimes followed peaks in the bacterial populations, numbers of bacteria were usually low during zooplankton maxima. For example, numbers of rotifers increased in October, 1970, but by this time the bacterial population had dropped considerably from the peak in September. However, in January, 1971, the bacterial and rotifer peaks coincided.

These results suggested that there was some correlation between bacterial and zooplankton populations, but little correlation between bacterial and phytoplankton populations. However, at all times the increases in the bacterial population were only small when compared with the numbers of zooplankton and algae which were a potential source of organic matter for the bacteria.

Association of bacteria and plankton

Three methods of concentrating the plankton or separating it from lake water were used to determine the proportion of the bacterial population which was directly associated with the plankton. Filtration through a 3 μm filter ensured that algae and zooplankton were retained on the filter. The results from filtering lake water from two depths, sampled on the same day, showed that the proportion of the bacterial population remaining on the filter varied, but that the majority of the bacteria passed through the filter (Table 7.2).

TABLE 7.2 Fractionation of the bacterial population by filtering through a 3 μm pore-size filter

Sample	No. bacteria (per ml)		Bacteria on filter (% of total population)
	in filtrate	on filter	
25/11/69 open water - 3 m	69 ± 5	24 ± 2	26
25/11/69 open water - 7 m	125 ± 15	6 ± 1	5

Concentrating the plankton, by passing one litre of lake water through a net, resulted in a sample of about 50 ml which included all the zooplankton from the lake water and some of the phytoplankton. From the bacterial plate counts of this concentrated plankton, the numbers of bacteria associated with plankton in one litre of lake water were calculated (A). However, the concentrated plankton sample included some water and, as well as any bacteria directly associated with the plankton, there would have been some free-floating bacteria in the sample. The plate counts of a separate sample of untreated lake water were used to determine the number of bacteria in a volume of lake water equivalent to that containing the concentrated plankton. This figure (B) was subtracted from (A) and the proportion of the bacterial population directly associated with the plankton in 1 ml of lake water was calculated.

The results for the sample collected on 23 November,

when more bacteria were found associated with the plankton in 1 ml of lake water than the total numbers of bacteria per ml, showed that variation in the numbers of bacteria from one sample to another could be a problem in determining (B). Apart from this sample, however, this method of calculating the numbers of bacteria associated with the plankton, provided an indication of the small part of the bacterial population which was associated with the plankton (Table 7.3). There was a slight increase in the percentage of the bacterial population associated with the plankton between 9 and 16 November. This coincided with the drop in numbers of rotifers.

When samples of plankton were obtained by centrifuging 10 ml of lake water, they consisted predominantly of phytoplankton. From the plate counts of the pellet fraction, the number of bacteria associated with the plankton in the 10 ml sample was determined (A). As with the samples of net-concentrated plankton, some water was included with the centrifuged plankton samples. The sum of the bacterial populations of the pellet and supernatant fractions was used to obtain an estimate of the numbers of bacteria in the original 10 ml sample. From this estimate, the number of bacteria in the volume of water containing the centrifuged plankton was calculated (B). After subtracting (B) from (A), the percentage of the total bacterial population in 1 ml of lake water, which was associated with the plankton, was calculated.

The samples collected on 7 December, 1970, were concentrated by passing them through a net and by centrifuging. The percentages of bacteria associated with zooplankton and

TABLE 7.3 Proportion of the bacterial population associated with plankton after concentration by passing through a net or by centrifugation

Date sampled	No. bacteria in lake water (per ml)	Method of concentration	No. bacteria associated with plankton (per ml)[1]	(as % of total population)
9/11/70	390 ± 85	net	42	11
16/11/70	680 ± 85	net	111	16
23/11/70	180 ± 45	net	222	-
7/12/70	310 ± 190	net	40	13
7/12/70	212[2]	centrifugation	30 - 32[3]	14 - 15[3]
5/ 1/71	519[2]	centrifugation	63	12
1/ 2/71	454[2]	centrifugation	69	15
24/ 2/71	545[2]	centrifugation	52	10
8/ 3/71	165[2]	centrifugation	0	0

1 Number of bacteria associated with plankton corrected as described in text to allow for bacteria in any lake water in the sample of concentrated plankton.

2 Number of bacteria per ml calculated from sum of bacteria in pellet and supernatant after centrifuging.

3 Pellet volume not recorded and correction estimated on basis of pellet volume of later samples.

phytoplankton, determined from the net-concentrated sample, and with mainly phytoplankton, obtained by centrifuging, were very similar (Table 7.3). With the decrease in volume of phytoplankton between 1 February and 24 February, there was a drop in the percentage of the population associated with the plankton. This drop was more marked in the sample collected on 8 March.

These experiments showed that a proportion of the bacteria in lake water appeared to be associated with the plankton. However, as shown by the samples collected in February and March, this fraction was related to the amount of plankton present.

Kinds of bacteria in samples of plankton and water

The kinds of bacteria in samples of net-concentrated plankton and lake water and, on one occasion, centrifuged phytoplankton, collected between 9 November and 7 December, 1970, were examined (Table 7.4). The bacteria found to be predominant in most of the samples were flavobacteria, coryneforms, pseudomonads and Vibrio extorquens. During the month in which bacteria were characterized, the numbers of zooplankton and phytoplankton decreased considerably. Between 9 and 16 November, there was an increase in the proportion of pseudomonads and Vibrio extorquens associated with the plankton, while numbers of flavobacteria decreased. At the same time, rotifer numbers dropped from 267 to 4 per litre. The bacterial population of the open water sampled on 16 November consisted mainly of Micrococcaceae, which were not found in such large numbers in any of the other samples characterized. This increased number of cocci in the water

TABLE 7.4 Kinds of bacteria in open water and associated with plankton

Date sampled	Sample	No. characterized.	Percentage distribution.											
			Alcaligenes/ Achromobacter	pseudomonads	flavobacteria	Cytophaga	Vibrio extorquens	Enterobacteriaceae	Aeromonas/ Vibrio	Acinetobacter	Chromobacterium	coryneforms	Micrococcaceae	Bacillus
9/11/70	Open water	27	+	34	22		7		7			22	+	
9/11/70	Plankton	25		+	47	+	7					25	13	
16/11/70	Open water	41	7	+	7	+						7	71	
16/11/70	Plankton	41		23	17		27		+			29	+	
23/11/70	Open water	38	+	21	52	+	11					12		
23/11/70	Plankton	41		+	85		12							
7/12/70	Open water	38	8	35	35	+		+		+		16		
7/12/70	Plankton	48		9	54							33		+

+ $\leq 5\%$

coincided with the drop in the rotifer population.

Although the phytoplankton population on 16 and 23 November remained at a moderate level, and the zooplankton population at a low level, the kinds of bacteria in both the untreated water and plankton samples changed considerably. However, these changes were emphasized because of the large proportion of cocci in the open water sample on 16 November and the predominance of flavobacteria in the plankton sample on 23 November. Apart from the sample of open water collected on 16 November, the composition of the bacterial population of the other three samples of untreated water was fairly similar. The three plankton samples, other than the sample collected on 23 November, however, did not have so much in common. The percentages of all the major groups found, except the coryneforms, fluctuated considerably between the three samples.

Vibrio extorquens was a particularly interesting group. For the samples collected on 7 December, when plankton was concentrated both by passing water through a net and by centrifuging, bacteria were characterized fully from the open water and net-concentrated plankton and, in addition, 50 bacteria were isolated on to GYCA from the two water fractions after centrifuging and their pigmentation noted. A selection of the distinctive pink organisms were Gram-stained and proved to be Vibrio extorquens. The proportions of this bacterium and also of pigmented bacteria as a whole in these samples are shown in Table 7.5.

The lack of Vibrio extorquens in the untreated sample from the open water may have been due to chance because of the number of bacteria characterized, but it is surprising

TABLE 7.5 Percentages of pigmented bacteria and Vibrio extorquens in samples collected on 7 December

Sample	No. characterized	Vibrio extorquens (%)	Chromogens (%)
Open water	38	0	45
Net-concentrated plankton	48	0	88
Centrifuged plankton	50	50	70
Supernatant	46	11	43

that this species made up such a large proportion of the sample of centrifuged plankton, but was not found in the net-concentrated plankton. This species is considered in more detail in Chapter 11.

From the analysis of the individual samples, the only group which was predominant as plankton numbers were decreasing was Micrococcaceae. However, when all the samples were considered together it was seen that flavobacteria, coryneforms and Vibrio extorquens were more common in the plankton samples than the untreated lake water (Table 7.6). Consequently, there were more pigmented bacteria associated with the plankton (Tables 7.5 and 7.6). The proportion of Gram-negative bacteria in both sets of samples was similar (Table 7.6).

TABLE 7.6 Kinds of bacteria isolated from open water and plankton, sampled in November and December, 1970

Sample	Total number characterized	Percentage distribution					
		pseudomonads	flavobacteria and Cytophaga	Vibrio extorquens	coryneforms	Microc-occaceae	others
Open water	144[1]	21	32	4	13	21	9
Net plankton	155[2]	10	52	11.5	22	2.5	2

Further division of flavobacteria and Cytophaga

Sample	Percentage distribution		
	flavobacteria[3]		Cytophaga
	motile	non-motile	
Open water	65	26	9
Net plankton	72	27	1

[1] Of these bacteria 65% were Gram-negative and 64% were pigmented.

[2] Of these bacteria 73% were Gram-negative and 83% were pigmented.

[3] Further characteristics of these two groups are given on p. 43.

Although 'flavobacteria and Cytophaga' was the predominant group in the plankton samples, when this broad group was separated further it could be seen that the proportions of motile and non-motile flavobacteria in the samples of plankton and untreated lake water were similar. Motile flavobacteria were more common than non-motile flavobacteria in both cases. However, a larger proportion of Cytophaga was found in the untreated lake water (Table 7.6). Pseudomonads made up a part of the microflora of both the open water and plankton but were less common in the samples of plankton. Fluorescent pseudomonads were rare in all the samples.

Bacteria associated with individual organisms

As certain groups of bacteria were found more frequently in the samples of plankton than in those of untreated lake water, it appeared that the plankton might have a specfic microflora. To study this further, individual planktonic organisms were placed on nutrient agar and incubated (Table 7.7).

No bacteria grew when animals had been washed before placing on the agar. Unwashed animals carried some lake water and algae on them, and some bacteria grew. The unwashed Bosmina and Keratella were not visible on the agar after incubation so it could not be determined whether the colonies had grown from bacteria on the animals. One colony did, however, grow on the unwashed Ceriodaphnia isolated on 7 December. There were no colonies on the Piona, which had crawled on the agar, but three colonies grew on the track made by the animal. Bacterial colonies always grew from

TABLE 7.7 Bacteria associated with individual organisms sampled in October, November and December, 1970, and December, 1971

Sampling date	Organism	Treatment	No. colonies	Kinds and comments
9/10/70	live *Bosmina*	not washed [1]	4	chromogens
5/11/70	dead *Bosmina*	3 washes	none	-
5/11/70	live *Bosmina*	3 washes	none	-
5/11/70	live *Ceriodaphnia*	3 washes	none	-
5/11/70	live *Keratella*	not washed	4	chromogens
5/11/70	algae	not washed	several	chromogens including *Vibrio extorquens*
7/12/70	live *Piona*	not washed, crawled 2 cm	10	3 on track, none on *Piona*
7/12/70	live *Ceriodaphnia*	2 washes	none	-
7/12/70	live *Ceriodaphnia*	not washed	3	1 coryneform on *Ceriodaphnia*
7/12/70	algae	not washed	28	25 chromogens - 1 *Vibrio extorquens*
5/12/70	algae	not washed	11	5 chromogens

[1] a drop of agar was placed on top of this animal

the algal samples and were mainly chromogens, but compared with the numbers of algae placed on the agar, the numbers of colonies were very small.

7.3 DISCUSSION

Estimation of quantity of algae

The estimation of the quantity of the algae in a field sample by counting the number of cells is complicated by the differences in sizes of cells. Paasche (1960) compared primary production and number, surface area and volume of cells of marine phytoplankton as estimates of the size of the standing crop. Primary production was estimated by the ^{14}C method. Paasche concluded that cell number or cell volume over-emphasized the role played in production by small or large algae, respectively. In the study of Lake Grasmere, the amount of algal material that was available for decomposition, rather than the primary productivity of the algae, was being considered, and thus volume was a measurement which reduced the over-emphasis of the small Cyclotella cells and gave a meaningful measure of algal biomass.

Measurements of the abundant algae over several months showed that, as expected with diatoms continually dividing, the dimensions of the cells changed (Appendix 6). This was most noticeable with Cyclotella. As few cells were measured there may have been considerable error in the data used to calculate cell volumes and surface areas of the algae at different times of the year. The assumption that Asterionella was a cuboid also introduced some error as many of these cells

were not a constant width in girdle view. Any error in the calculations for one cell would be increased when the volume of algae per ml of lake water was calculated. However, such errors would be small in comparison with the marked fluctuations which occurred in the algal populations.

The range of volumes of Asterionella (Table 7.1) was within those reported by Goldman et al. (1968), Ruttner (1959) and Nauwerck (1963). The volumes of Diatoma and Melosira (Table 7.1) were smaller than the volumes of these algae in Lake Erken measured by Nauwerck (1963).

Seasonal variation in plankton and bacterial populations

The data presented in Fig. 7.3 were examined in detail in an attempt to determine whether the bacterial population was strongly influenced by the plankton populations, and also whether the zooplankton populations were responding to fluctuations in the bacterial population. Because the three groups within the plankton - rotifers, Cladocera and phytoplankton - were all influencing the bacteria at the same time although probably in different ways, it was difficult to determine the causes of any fluctuation in the bacterial population. The problems of sampling zooplankton had also to be taken into account. Migration of animals from one depth to another may result in unusually low or high counts. With such a low count it might be inferred that the animals had died and become available to bacteria for decomposition when in fact they were alive, but at another depth. Although numbers of zooplankton from one depth only are reported here, counts throughout the water column

were made and were checked to see whether the count at three metres was representative. They were found to be representative apart from the very low numbers of zooplankton recorded at 3 m on 3 May when, at 5 m, more than 150 rotifers were found in the litre sample.

At the beginning of November, the numbers of rotifers decreased and at the same time there was a sudden increase in numbers of bacteria. This increase could have resulted from a decrease in grazing by rotifers, or could have been in response to the death of the large population of rotifers. As the numbers of bacteria were not very high in October when the rotifer population increased, it is unlikely that bacteria were a major part of the rotifers' diet, but some depletion of the bacterial population may have occurred when rotifer numbers were at their peak. The main response by the bacteria may therefore have been to products from the dying zooplankton.

Bacteria may have had a major influence on zooplankton populations in September. The large bacterial populations recorded at this time might well have stimulated egg production among the rotifers present and have been one cause of the large rotifer maximum in October. Although no information is available for the rotifers, there was greater egg production in the Cladoceran - Ceriodaphnia dubia - in culture when bacteria were included in the diet. (Staples, unpublished). The increase in bacterial numbers in November was only short-lived and at the same time there was an increase in the numbers of Cladocera. If Cladocera were consuming a similar range of particulate matter to the rotifers, their numbers might well increase as rotifer numbers de-

clined. The role of bacteria in this increase is not clear, but as noted above egg production in culture is enhanced by the presence of bacteria and it is possible that the Cladocera were also responding to the large bacterial population in September but with a longer lag period. However, an analysis of data on egg ratios and instar periods, which was beyond the scope of this thesis, would be required before any such role could be established conclusively. The second increase in numbers of Cladocera, which was recorded at the beginning of December, followed the peak in numbers of bacteria in November.

As the increase in numbers of bacteria and Cladocera was occurring in November, the diatom population was decreasing considerably. There appeared to be little response by the bacteria to the death of so many algae except for the slight increase in bacterial numbers in December. With the large population of Cladocera found in December, the response by bacteria to the death of the algae might have been masked by grazing of bacteria by these Cladocera. A sample taken between 23 November and 7 December might have detected a greater fluctuation in the bacterial population.

The increased numbers of bacteria at the beginning of January could have been in response to death of Cladocera. These animals decreased in numbers as both rotifer and algal populations increased. Also, in response to some factor such as a nutrient, algal, bacterial and rotifer numbers might all have increased but the bacteria, in competition with the algae possibly for trace elements or vitamins, did not remain at a high level.

This examination of the fluctuations in plankton and bacterial populations in November and December, 1970, showed that to detect responses by bacteria to changes in plankton populations, sampling at close intervals as was done in November, 1970, was necessary. The data suggested that bacteria utilized dead rotifers and probably Cladocera, but a response to dead algae was not marked. On the other hand, it seemed likely that bacteria enhanced egg production but it was less clear whether zooplankton were grazing bacteria to any extent. The data from the remainder of the samples from February to July confirmed these hypotheses.

The increased number of bacteria found at the end of February after the summer algal bloom was very small in comparison with the large population of Melosira and Cyclotella which existed in January and February, and no response could be distinguished after the algal bloom of Diatoma in April. In fact, near the end of each algal bloom, numbers of bacteria decreased e.g. 5 November, 1 February and 3 May. The increased number of bacteria in late February, while possibly indicating some response to declining algal numbers, may also have been related to the decrease in the rotifer population. However, no similar response was detected following the rotifer peak in April. This may have been due to the smaller number of rotifers present.

It should be noted that during late February and March, when a bacterial response to the death of Melosira and Cyclotella would be expected, both rotifer and Cladocera numbers were very low. Therefore, it is unlikely that grazing by zooplankton was masking any bacterial response at this time.

Between February and July, Cladocera were only found in small numbers so no bacterial response to the death of a large population of these animals could be examined. The increased bacterial population in June could have been related to the drop in the rotifer population which occurred at this time and may have been one factor responsible for the peak in numbers of Cladocera in late June.

This analysis has shown the problems of interpreting causes of fluctuations in populations of several interrelated organisms. It should be emphasized that the large increase in numbers of bacteria at the beginning of January could be related to a number of factors. Similarly, the cause of the peak in the bacterial population in February can only be suggested. The most significant results of this investigation were the lack of a marked increase in numbers of bacteria after diatom blooms and the small response by bacteria to zooplankton blooms.

Some similar responses have been reported. The study of Henrici (1938) is noted for the three bacterial peaks which followed two diatom blooms and a blue-green algal bloom in a eutrophic lake. A close examination of this data showed that there was a longer lag phase in the response of bacteria to the diatom blooms and after one such bloom, algal numbers had dropped to a very low level before bacterial numbers increased. It is possible that these increases in the bacterial population were really related to fluctuations in zooplankton populations, but Henrici does not mention these in relation to the diatom blooms. Comparing the response of bacteria to blue-green algae and diatoms, Taylor (1949), and Collins

(1957) in a further report on similar work, noted that there was no significant increase in plate counts of bacteria following the death of large numbers of Asterionella, but after a blue-green algal bloom there was a marked increase in the bacterial population. Jones (1971) concluded that there was no simple relationship between algae and bacteria, and Gerletti and Melchiorri-Santolini (1968) were unable to find any significant correlation between fluctuations in bacterial and algal populations. Goldman et al. (1968) doubted the correlation they found between plate counts of bacteria and phytoplankton populations and the complexity of the relationship was confirmed by Štěpánek (1968).

Increases in the numbers of bacteria after an algal bloom, as recorded by Henrici (1938) and Schegg (1968), would appear to be confined to eutrophic lakes. This may be because of the more readily decomposable algae predominating in these lakes or because of the presence of nutrients which, in less nutrient-rich lakes, may be limiting bacterial utilization of algae. However, even in eutrophic lakes there may be no marked increase in bacterial populations following an algal bloom. Silvey and Roach (1964) found that Gram-negative heterotrophs were at the highest level before a diatom bloom but they did not detect any marked increase in numbers of these bacteria after the bloom. They noted, however, that Gram-positive heterotrophs were more numerous after the diatom bloom.

A further complication in the elucidation of the relationship between bacteria and phytoplankton is the kinds of bacteria which are included in the studies. Where the total bacterial population has been estimated by a direct

count, the correlation with phytoplankton populations is sometimes different from the correlation between cultured bacteria and phytoplankton. Thus, Goldman et al. (1968) found a negative correlation between the phytoplankton population, which was made up mainly of diatoms, and the total bacterial population, although they recorded a positive correlation between cultured bacteria and phytoplankton. However, in a study by Overbeck and Babenzien (1964), in which diatoms were also the dominant algae, total numbers of bacteria were often at their peak at the same time as the algal maxima, but there was rarely a response by these bacteria to the decrease in numbers of algae.

It is clear from the results presented here and from the previous studies that there is not a simple relationship between the growth of algae and their decomposition by bacteria. One of the many other factors which must be taken into account is the role of zooplankton. Grazing of bacteria by zooplankton was suggested by the fluctuation in plankton and bacterial populations studied by Fondén (1969a) and Drabkova (1965). In a study of the bacteria and phytoplankton of Narrangansett Bay where the diatom Skeletonema costatum was predominant, Sieburth (1968) was unable to detect any increases in the bacterial population which might correspond with the seasonal outbursts of the diatom. He considered that zooplankton must be grazing bacteria.

In Lake Grasmere the bacterial population may be reduced by zooplankton. However, as there was only a small bacterial response in February to the summer algal bloom, although zooplankton was sparse, it seems unlikely that zooplankton is a major factor limiting the numbers of bacteria

in Lake Grasmere.

Association of bacteria and plankton

As well as studying the response of bacteria to fluctuations in plankton populations, the association of bacteria with the plankton was examined (Tables 7.2 and 7.3). These experiments indicated that the proportion of the bacterial population in lake water associated with the plankton was small, although there were limitations in the methods used. The pore-size of the filter was small enough to retain all algae and zooplankton but a number of bacteria could also have been retained. Experiments with a pure culture of a pseudomonad showed that most of these bacteria did not pass through the filter. If only a small proportion of the population had passed through the filter, few conclusions could have been drawn, as the size of the bacteria might have been limiting their passage. However, with the field samples, the majority of the bacteria passed through the filter and from this it can be concluded that only a small proportion of the bacterial population was either too large to go through the filter or was attached to the plankton. In the experiments where plankton was concentrated by passing through a net or by centrifuging, the correction for bacteria in lake water in the samples did not allow for any bacteria on plankton in such a sample, but this would increase the percentage associated with the plankton by only a small amount. In fact, the small proportion of the bacteria associated with the plankton may be an overestimate, as suggested by the results from the sample collected on 8 March when the amount of phytoplankton

in the sample was small. It is possible that the apparent association of bacteria and plankton was due to a coincidental lodging of bacteria on plankton as it was concentrated.

The numbers of bacteria in samples of marine plankton, which were concentrated by passing sea water through a net, have been estimated by several workers (Waksman et al., 1933; Rigomier, 1967; Seki, 1967; Simidu, Ashino and Kaneko, 1971). Unfortunately the proportion of the bacterial population that was associated with the plankton in situ was not calculated in these studies. Although large numbers of bacteria were found in the concentrated plankton, if the amount the sample had been concentrated had been taken into account, it might have been found that only a small part of the bacterial population was associated with the plankton in situ.

Kinds of bacteria in samples of plankton and water

The kinds of bacteria in the samples of lake water and concentrated plankton were examined to see whether there were any differences. Pigmented bacteria, including flavobacteria were more abundant in the plankton samples, and Vibrio (apart from Vibrio extorquens) was rare in the samples of both water and plankton (Tables 7.4 - 7.6). These results are similar to those of Sieburth (1968), who found that Flavobacterium was associated with dominance of a marine diatom while Vibrio appeared to be inhibited by large numbers of the diatom. Although some of these plankton samples did not include all the algae in the original water sample, a large proportion of the phytoplankton population was included.

Flavobacteria, and motile flavobacteria in particular, appeared to be predominant in the plankton samples from Lake Grasmere. This can be compared with the predominance of non-motile flavobacteria in the samples of Elodea, noted in Chapter 6.

Other groups of bacteria which were more common in plankton samples were coryneforms and Vibrio extorquens. The role of Vibrio extorquens is examined in more detail in Chapter 11. The larger proportion of coryneforms associated with the plankton may be related to the decrease in phytoplankton populations as noted by Silvey and Roach (1964). However, there was no marked increase in the numbers of this group as the diatom bloom ended. The only group of bacteria which became more common as zooplankton numbers decreased was Micrococcaceae. A few of these cocci, which were mainly yellow or orange, were found in the plankton sample when rotifer numbers began to drop and they made up a large proportion of the bacterial population of the water when few rotifers were left. It is possible that this bacterial group had responded to products from the dying zooplankton.

The almost complete lack of Vibrio (apart from Vibrio extorquens) in the samples of both plankton and water examined here does not confirm the results of Simidu, Ashino and Kaneko (1971). However, they mention that the concentration of plankton in the water was not large. They also found a slightly larger proportion of flavobacteria in the phytoplankton samples than in the water but this group made up only a very small part of the water population. The predominance of Vibrio in their plankton samples may have been a reflection of the environmental conditions which were

influencing the composition of the bacterial flora of the sea water.

The characterization of the bacteria from the samples from Lake Grasmere suggested that the presence of plankton influenced the abundance of certain groups of bacteria. However, the experiments in which the microflora of individual organisms was examined indicated that few if any of these bacteria were attached to the plankton (Table 7.7). These observations confirmed the results of the experiments in which plankton was concentrated or separated from lake water, which showed that only a small proportion of the bacterial population might be closely associated with the plankton (Tables 7.2 and 7.3).

In general, there was a marked lack of response by the bacteria in the open water to fluctuations in their major source of organic matter, and also a lack of any close association between bacteria and the plankton. Possible reasons are that:

a) bacterial decomposition of dead zooplankton and phytoplankton is slow so that there is little response by bacteria in the water before the organisms either sediment out on to the mud or float to the surface of the water;

b) bacteria are not the main agents involved in the mineralization of nutrients from dead zooplankton and phytoplankton;

c) live zooplankton and algae are unsuitable for colonization;

d) monitoring the changes in bacterial numbers by the plate count method may neglect a part of the bacterial

population which is more closely associated with the plankton populations.

The rate of bacterial colonization of algae and the possibility that algae have a specific microflora which has not been cultured are considered in Chapter 8. In Chapter 9, where the bacterial population of the mud is considered, the possibility that the main site of decomposition and recycling of nutrients is in the mud is examined. The importance of bacteria in the recycling of nutrients in general in the lake is considered in Chapter 12.

7.4 SUMMARY

1) The ratio between the numbers of algal cells and viable bacteria in the lake water varied from 4:1 to 34:1, with algae always outnumbering viable bacteria. The dominant algae were diatoms.

2) When two samples of lake water were filtered through 3 μm pore-size filters, 95% and 74%, respectively, of the bacterial population passed through the filter, showing that only a small part of the population was either attached to particulate matter, or had cells too large to pass through the filter.

3) Numbers of viable bacteria associated with plankton per ml of untreated water were calculated, and it was found that only between 0% and 16% of the bacterial population was associated with the plankton.

4) No bacteria were found on zooplankton which had been washed in sterile water and then plated on nutrient

agar. Up to 30 colonies grew from drops of concentrated algae, but the numbers of colonies were very small compared to the numbers of algae placed on the agar.

5) Some increase in numbers of bacteria in the lake water, as zooplankton populations declined, was detected, but there was little if any response to the death of large numbers of algae.

6) Bacteria, isolated from four pairs of samples of plankton, concentrated in a net, and untreated water, were characterized. Vibrio extorquens, flavobacteria and coryneforms were more common in the plankton samples, while pseudomonads and Micrococcaceae were more abundant in the untreated water. In samples of both untreated water and plankton, motile flavobacteria were more common than non-motile flavobacteria.

CHAPTER 8

BACTERIAL COLONIZATION OF ALGAE

In the previous chapter it was noted that algae always outnumbered bacteria when the latter were estimated by the pour plate method. Few if any bacteria, directly associated with the plankton, could be demonstrated by cultural methods. Plate counts of bacteria did not change markedly when the algal population increased or decreased. Several hypotheses concerning this lack of a relationship between bacteria and phytoplankton were made. It was suggested that living algae might be unsuitable for bacterial colonization and dead algae might be only slowly attacked so that little decomposition occurred in the lake water. Alternatively there might be a specific microflora which had not been cultured. It was, therefore, of interest to study the phytoplankton more closely to determine how readily algal cells were colonized by bacteria.

Although it is stated categorically in one recent publication that '... algal cells never are found in nature in the absence of intimately associated bacteria ...' (Dugan, 1972) there are a number of reports to the contrary. Steemann Nielsen (1955) observed that healthy, marine phytoplankton were apparently unsuitable for bacterial growth. This observation has been confirmed for algae in freshwater (Saunders, 1959; Overbeck and Babenzien, 1964), sea water (Droop and Elson, 1966), marine sediments (Oppenheimer and

Vance, 1960) and in culture (Sieburth, 1968). Waksman, Stokes and Butler (1937) studied the relationship between Nitzschia closterium and bacteria in culture experiments and found that the living diatoms were not attacked by bacteria. The green alga Scenedesmus was also resistant to bacterial attack when alive (Golterman, 1968).

However, while healthy algae are generally free of bacteria, there are exceptions. Saunders (1959) examined a number of diatoms, blue-green, and green algae and found that occasionally epiphytic bacteria were present. An Oscillatoria tenuis filament with two bacteria attached perpendicularly illustrated this. Overbeck and Babenzien (1964) found that bacteria were occasionally attached in small groups to freshwater diatoms. Examination of Tabellaria fenestra showed that only living cells had a bacterial flora while the empty frustules were free of bacteria. Taylor (1949) reported that a proportion of the cells of Asterionella formosa had rod-shaped bacteria attached at right angles to the cells. Localized concentrations of rod-shaped bacteria presumed to be embedded in zoogloea were also noted. Spencer (1952) in a study on the culturing of bacteria-free algae noted that the bacteria normally associated with algae, especially diatoms, were very tenaciously attached.

Much of the evidence for stating that bacteria are attached to both phytoplankton and zooplankton has been obtained from studies in which samples of plankton were first concentrated and then the numbers of bacteria in the samples were determined (Waksman et al., 1933; Rigomier, 1967; Seki, 1967; Simidu, Ashino and Kaneko, 1971). However, as noted in the previous chapter, the numbers of bacteria

associated with the plankton *in situ* were not considered in these studies.

The identity of bacteria, epiphytic on healthy algae, has received little attention. Taylor (1949) found that, although *Asterionella* had a bacterial flora which was possibly associated with the mature and moribund cells, there was little increase in the plate counts of bacteria at the end of an *Asterionella* bloom. This suggested that few of the epiphytic bacteria were being estimated by the plate method.

The morphology of the bacteria that were attached at right angles to the diatom cells was similar to *Caulobacter*. Apart from these observations, there is no data to indicate whether most epiphytic bacteria are similar to those included in plate counts of aquatic bacteria or are like *Caulobacter*.

In her study, Poindexter (1964) showed that *Caulobacter* cells could attach to a number of kinds of algae and that less healthy cells were attached to preferentially. *Caulobacter* has been reported attached to the bacterium *Leucothrix* (Hirsch, 1968). Other bacteria which attach to algae, but are however cultured on standard media, are the pathogenic flexibacteria which lyse blue-green algae (Daft and Stewart, 1971; Shilo, 1971). There is also the possibility that many of the epiphytic bacteria could be dead. This is not confirmed by the work of Meadows (1971). He found that only bacteria killed by UV radiation, in which the integrity of the cell wall was unaltered, attached as readily as live bacteria to glass and he concluded that under natural conditions bacteria killed by most forms of physical and chemi-

cal stress would be unable to attach to surfaces.

Several studies have been made on the toxic extracellular products of algae and also on the factors influencing the attachment of bacteria to mainly inanimate objects such as glass. Extracts of algae have been shown to be bactericidal (Duff, Bruce and Antia, 1966; Sieburth, 1968; Harris, 1971). Levina (1964) reported antagonisms between algae (Scenedesmus spp. and Chlorella vulgaris) and certain Enterobacteriaceae in culture. Sieburth (1959) found that Antarctic marine phytoplankton, which had been concentrated on a membrane filter, inhibited the growth of bacteria streaked across the other side of the filter. Changes in surface tension will influence bacterial attachment. Some production of surfactants, the amount of which differed among the species, has been demonstrated in five marine diatoms (Wilson and Collier, 1971).

An acid microzone near diatom cells (Sieburth, 1968) and the electrolyte concentration around a glass surface (Marshall, Stout and Mitchell, 1971) have been throught to control attachment of bacterial cells. Attachment is also influenced by the presence of proteins in the external solution (Meadows, 1971) and the level of nutrients (Ruschke, 1968; Marshall, Stout and Mitchell, 1971). Sorption of a Pseudomonas sp. was increased by 7 mg/l glucose but higher concentrations inhibited it (Marshall, Stout and Mitchell, 1971). Ruschke (1968) found that an aquatic myxobacterium attached to cellulose particles, to living and dead Chlorella and to yeasts in sterile filtered lake water, but failed to do so in a medium of carbohydrate and peptone.

Apart from factors in the external environment of the

algal cell, the physiological state of the cell is a major factor influencing bacterial attachment. Oppenheimer and Vance (1960) found a marked change in the susceptibility of algae to bacterial attack when they died. Observations on algae in marine sediments showed that almost all living algae were bacteria-free but that 89 of 91 dead diatoms had 1 to 25 bacteria absorbed to the surface or inside the frustule. These authors also noted that bacterial attachment was typically at right angles to the algal cell and suggested that this indicated a polar effect. In culture experiments with diatoms and bacteria, Waksman, Stokes and Butler (1937) found that dead diatoms were rapidly decomposed by bacteria although the live algae were not attached, and Golterman (1968) reported that a Pseudomonas could only utilize Scenedesmus cells once these had been killed and lysed.

Droop and Elson (1966) found that as the numbers of the marine diatom Skeletonema costatum declined, the numbers of attached bacteria increased to about ten per cell. Taylor (1949) reported that the appearance of bacteria on Asterionella formosa coincided with the maximum growth of the cell.

Summarizing these observations, it would appear that bacteria may be present on algae at all stages of growth but they are usually rare on healthy cells and may in some cases only be found on dead algae. Such observations raise the question of the role of bacteria epiphytic on living algae. Are they contributing significantly to the recycling of nutrients bound in algal cells? Are the factors influencing the attachment of bacteria to dead algae affecting the rate at which nutrients in algae are released?

In this chapter direct observations of algae for epiphytic bacteria and attempts to culture epiphytic bacteria are

described. The ability of bacteria isolated from Lake Grasmere to attach to a diatom in culture is considered and the significance of colonization of algae by bacteria is discussed in relation to the decomposition of algae.

8.1 METHODS

Direct observations of field samples

Samples were collected from the open water and from the littoral zone of the southwest and, on one occasion, northwest ends of the lake, between November, 1970, and March, 1972. They were centrifuged at 650 g for 5 min and wet mounts of the concentrated algae were examined with phase-contrast illumination for any epiphytic organisms. Littoral mud from the northwest end of the lake, sampled in February, 1972, was also examined. The sampling dates are given in Appendix 8.

Culturing epiphytic bacteria

Samples of lake water in which direct observation had shown the presence of epiphytic bacteria were used as inocula.

a) Ten ml samples were centrifuged at 160 g for 5 min and the concentrated algae were spread on the following media:

Exp. 1 nutrient agar - formula described on p. 26. Plates were inoculated within 5 h of sampling and incubated at 20°C in the dark.

Exp. 2 (i) caseinate agar - formula described on p. 26.

(ii) 'lake water agar' (LWA) - agar (1.5%) was added to lake water collected at the same time as that used as inoculum, and the medium was autoclaved.

(iii) 'filter-sterilized lake water agar' (FLWA) - lake water from the same sample as (ii) was filter-sterilized before agar was added and the medium autoclaved.

In Exp. 2 half of each plate was spread with 0.3 ml of a 'nearly bacteria-free culture' of Diatoma elongatum (p.60), killed by autoclaving. Centrifuged algal samples from lake water which had been kept at 5°C overnight were streaked on to both halves of the plates and incubated at 20°C in the dark for 12 days. Plates were examined for colonies and samples of the autoclaved algae were examined with phase-contrast illumination for epiphytic bacteria.

b) Three ml of a 'nearly bacteria-free' culture of Diatoma elongatum, which had been growing for 5 weeks, was inoculated with 0.5 ml of lake water. The tube was incubated at 17°C with 16 h light and 8 h dark in the algal culture room, as described on p. 57. Samples of the algae were examined with phase-contrast illumination after 2 and 14 days for epiphytic bacteria.

c) The possibility that bacteria epiphytic on healthy algae were Caulobacter spp. was considered. Lake water from near the shore was enriched with 0.01% peptone as described by Poindexter (1964) and incubated at room temperature. Samples were examined with phase-contrast illumination at intervals for up to 16 days and loopfuls of bacterial suspension were streaked on to a dilute peptone medium (Poindexter, 1964) and incubated at 30°C. The samples used as inocula in these experiments are described in Appendix 8.

Comparison of epiphytic bacteria and cultured bacteria

a) Uptake of radioactive compounds

Lake water sampled in March, 1971, was filtered through a 10 μm and then a 0.45 μm pore-size Millipore filter. The concentrated microorganisms were labelled with ^{3}H-glucose or ^{3}H-thymidine, scraped from the filters on to glass slides and autoradiograms prepared as described on pp. 80 and 82. Autoradiograms were made of another sample of water, which was collected in January, 1972, and kept at 5°C for 24 h before labelling. Ten ml of this sample was centrifuged for 5 min at 160 g. The concentrated suspension was labelled with ^{3}H-glucose and autoradiograms prepared as described on pp. 80 and 82.

b) Attachment of bacteria isolated from Lake Grasmere to 'nearly bacteria-free' Diatoma elongatum in culture.

The following bacteria isolated from Lake Grasmere were tested:- Micrococcaceae - 3D 11; pseudomonads - 6E 9, 6D3 7, 6W 5; coryneform - 6W 24; Enterobacteriaceae - 6D3 3; flavobacterium - 6D3 33; Cytophaga - 13P 1; Vibrio extorquens - 14P 39.

(i) Tubes containing one ml of a young culture of Diatoma (9 days old) were inoculated with a loopful of one of the test cultures which had been growing in nutrient broth. Wet mounts were examined for attached bacteria after 15 to 60 min at room temperature and after 48 h at 17°C in the algal culture room.

(ii) Three ml of Diatoma culture (15 days old) were inoculated with Vibrio extorquens from a nutrient agar slant and incubated in the algal culture

room. Samples of algae were examined after 1, 4, 6 and 18 days.

(iii) A culture of Diatoma which had been growing for 15 days was harvested by centrifugation. The cells were resuspended in autoclaved tap water or fresh algal medium (p. 56). For each test isolate a loop of a 48 h bacterial culture from a nutrient agar slant was suspended in autoclaved tap water. The algal suspensions were inoculated with a loop of the crudely-washed bacteria. All the isolates listed above were tested except for Vibrio extorquens. Wet mounts were examined with phase-contrast illumination after 18 h and 4 days.

c) Bacterial population of bacterized algal cultures

(i) Wet mounts of algae from cultures of both the longer taxon and shorter taxon of Diatoma elongatum were examined for epiphytic bacteria.

(ii) A loopful of culture from the longer taxon of Diatoma elongatum which was bacterized was added to a suspension of the shorter 'nearly bacteria-free' taxon. The tube was incubated in the algal culture room for 7 days and then left at room temperature to encourage bacterial colonization. The suspension was reinoculated with the longer taxon culture after 14 days and incubated in the algal culture room. Samples of algae were examined at intervals for up to 28 days after the first inoculation.

Possibility of formation of toxins by Diatoma elongatum

A method similar to that described by Sieburth (1959) was used. A 16 day-old culture of 'nearly bacteria-free' Diatoma elongatum, in which direct examination had shown all the cells were healthy, was harvested by centrifugation. Millipore filters (pore-size, 0.45 μm; diameter, 47 mm) were cut in half and placed on to plates of FLWA (p.252). A drop of concentrated algal suspension was placed on one half only of the half filters. When the filters had dried they were inverted and a loop of bacteria from a nutrient agar slant was streaked once across the half filter. Four isolates were tested : - Vibrio extorquens, Cytophaga, Enterobacteriaceae and a fluorescent pseudomonad. After incubation at 20°C for 10 days, the amount of growth on the filters was assessed.

8.2 RESULTS

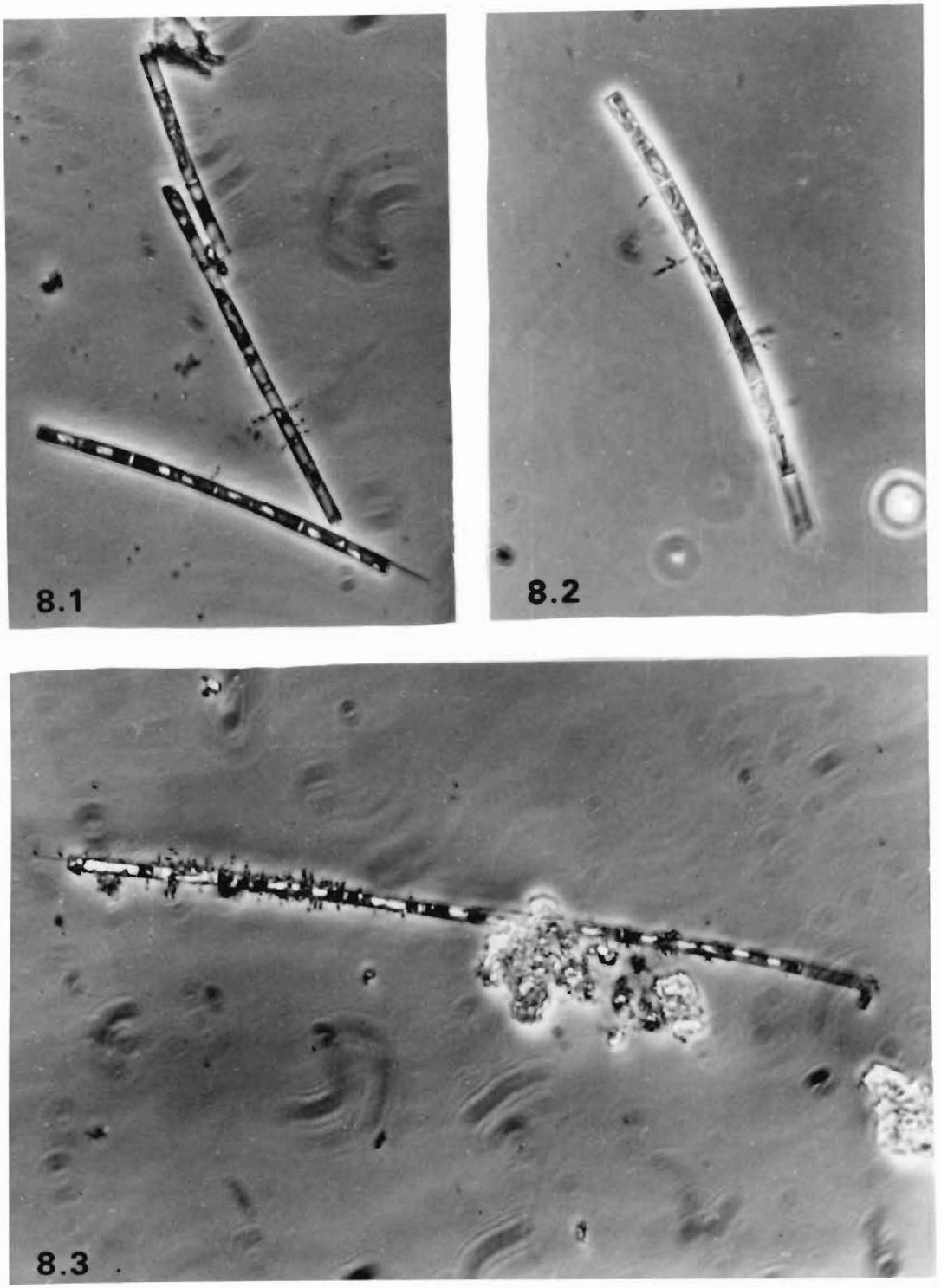
Direct observations of field material

Algae from the lake were examined directly during the summers and autumns of 1970/1971 and 1971/1972. The dominant algae and the abundance of epiphytic bacteria both varied considerably during the two periods.

Between November, 1970, and April, 1971, the following algae were observed as they increased and decreased in numbers:- Diatoma elongatum, Asterionella formosa, Melosira granulata var. angustissima and Cyclotella kůtzingiana. Some healthy filaments of Mougeotia sp. and colonies of other green algae, such as Eudorina elegans, were also examined.

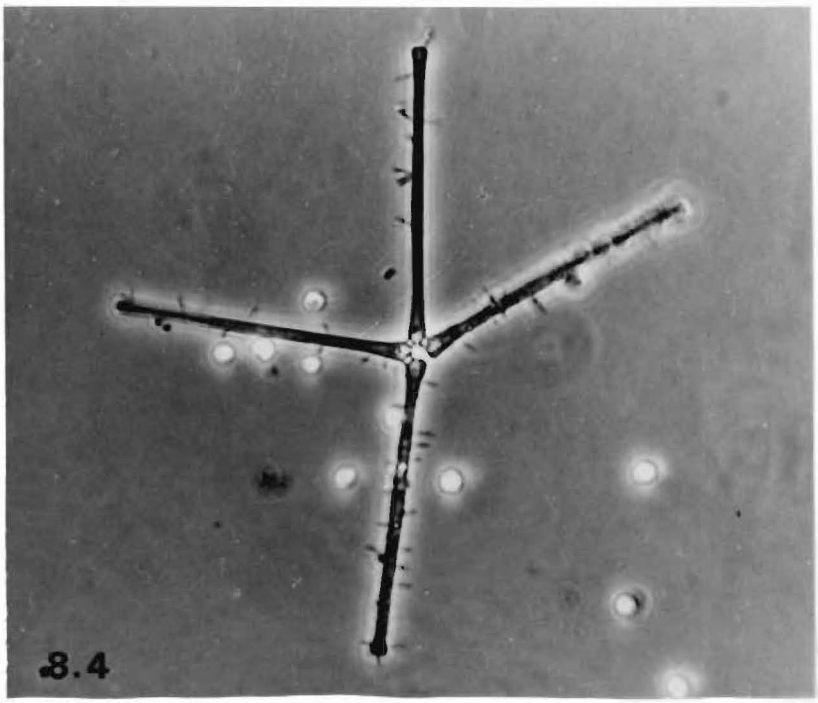
The only alga, on which epiphytic bacteria were seen, was Melosira. In March, 1971, when the number of Melosira cells per ml had dropped to one fifth of the maximum number recorded, there were occasional epiphytic bacteria on some of the filaments (Figs. 8.1 and 8.2). Many of these bacteria were in short chains and all were attached at right angles to the algal cells. Two weeks later when there were only a few filaments of Melosira in the sample some filaments had large numbers of bacteria on them (Fig. 8.3) but others were bacteria-free. These bacteria were only found on Melosira when the bloom was declining and, in the 1970/1971 season, apart from the bacteria on Melosira, epiphytic bacteria were not seen.

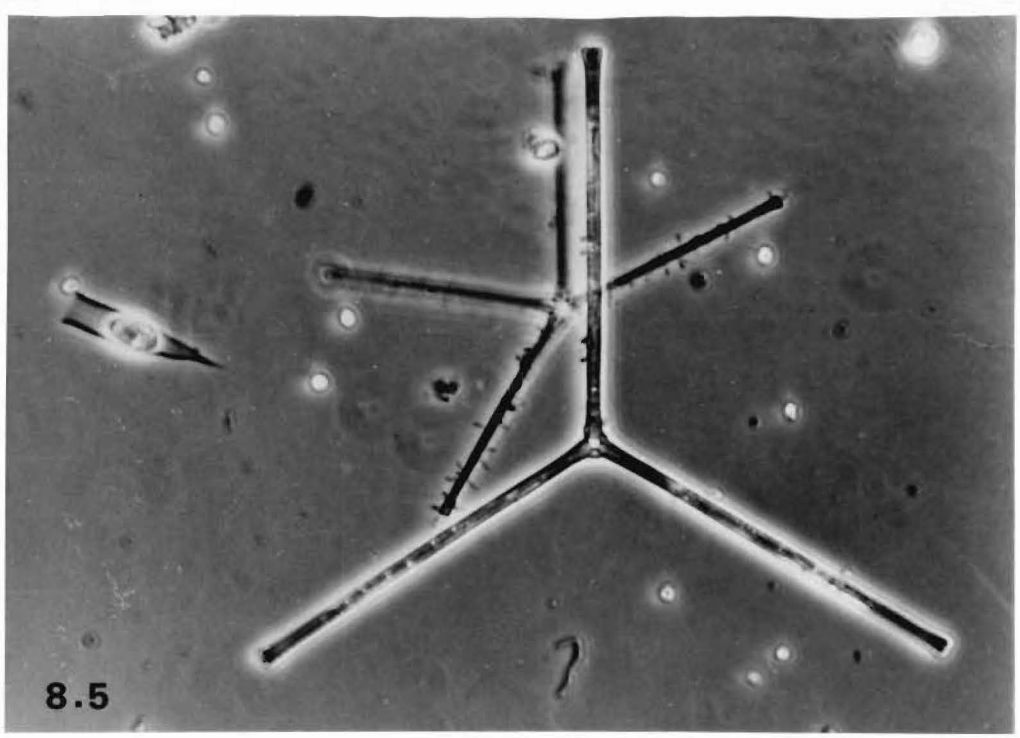
In the summer of 1971/1972, Asterionella was far more common than it was in the previous year and Melosira was not so abundant. Apart from Asterionella, the main algae found in December, 1971, were Fragilaria crotonensis, Diatoma elongatum and Dinobryon sp.. A Chrysophycean sp. was present on some of the Asterionella cells. Data obtained from Dr E.A. Flint showed that during October and early November, Asterionella was very common. It is likely that, when the sample was examined at the beginning of December, Asterionella had passed the point of maximum growth although many healthy colonies, mostly containing eight cells, were still present. In December, 1971, nearly all cells of Asterionella had unicellular bacteria attached perpendicularly. Fig. 8.4 shows epiphytic bacteria on cells of a colony of four cells as focussing on more than one or two cells of the larger colonies was difficult. Epiphytic bacteria were also common on Fragilaria, but were seen on only occasional cells of Dinobryon and Diatoma (Fig. 8.5). These epiphytic bacteria were



Figs. 8.1-8.3 Filaments of Melosira granulata var. angustissima, showing different amounts of bacterial colonization.

Fig. 8.1 - sampled 8 March, 1971; x 600.
Fig. 8.2 - sampled 8 March, 1971; x 700.
Fig. 8.3 - sampled 22 March, 1971; x 600.





FIGS. 8.4 and 8.5 Algal cells showing different amounts of bacterial colonization; sampled December, 1971.

Fig. 8.4 - Asterionella formosa; x 700.

Fig. 8.5 - Asterionella formosa, Diatoma elongatum, Dinobryon sp.; x 580.

found on both healthy and moribund algal cells.

Samples of lake water were then examined at two-weekly or monthly intervals until March, 1972. During January, 1972, the numbers of Asterionella declined and most of the colonies contained only four cells. Almost all healthy cells still had bacteria on them, but there were fewer per cell. However, there tended to be more bacteria on the moribund cells than on the healthy cells. At the same time, numbers of Diatoma and Melosira increased but only a few cells of these diatoms had an epiphytic flora. In March, 1972, the numbers of Asterionella and Fragilaria again increased, but apart from one Asterionella cell with a single bacterial epiphyte, no bacteria were visible on these algae. There were, however, a few epiphytic bacteria on some Melosira filament fragments. These bacteria were similar to those seen in the previous year and illustrated in Figs. 8.1 - 8.3.

In samples of water from the littoral zone collected in December, 1971, and January, 1972, there was a large unicellular diatom - Synedra ulna (Nitzsch) Ehrb. - which was not found in the open water samples. Almost all cells of this diatom had a few large bacteria attached, often in a chain of two cells (Fig. 8.6), and some cells also had many slender bacterial epiphytes. These bacteria appeared to be quite different from the epiphytes on Asterionella and Melosira and occurred on both healthy and moribund cells.

In February, 1972, a littoral mud sample was examined and it was found that algal fragments and most live algae were without a bacterial flora. A few epiphytic bacteria were visible on some live cells of a Nitzschia sp..

Culturing of epiphytic bacteria

a) On agar media

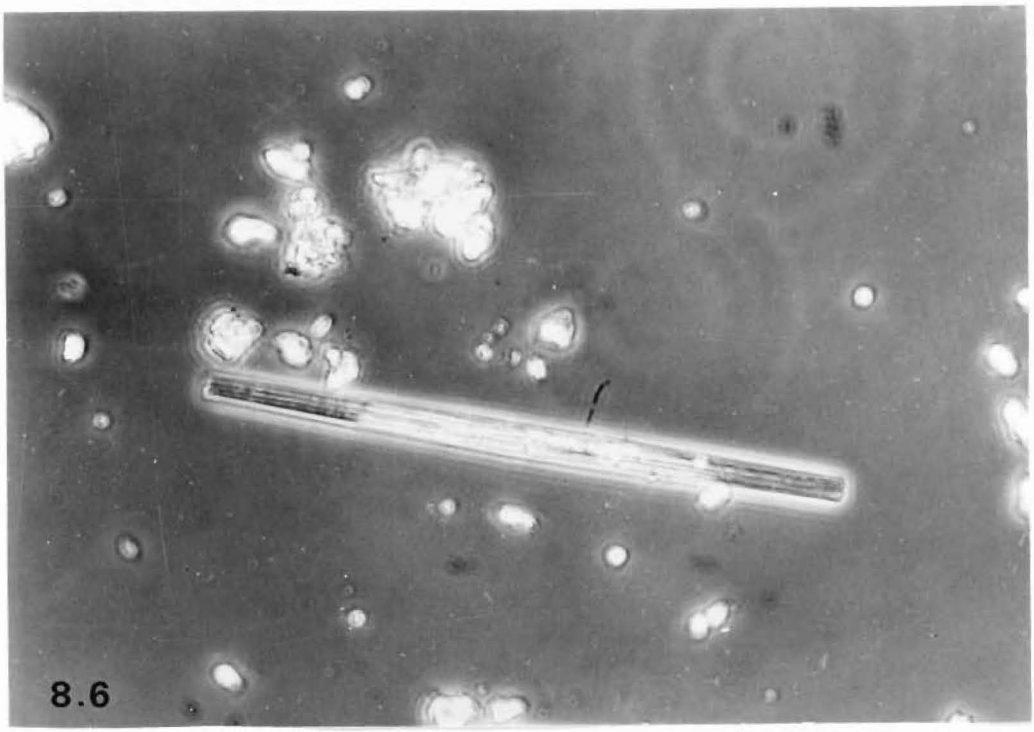
Samples collected during December, 1971, when epiphytic bacteria were common on Asterionella and also, in the littoral sample, on Synedra ulna, were used as inocula. Only 11 colonies grew on nutrient agar. More colonies grew on the other media inoculated with the sample of open water, but if these had originated from the epiphytic bacteria, only very few had formed colonies. Few colonies grew on the plates of the littoral algae. Examination of the algal cells, which were spread on to the plates before inoculation, showed that, while many were bacteria-free, others had a number of bacteria around them. These bacteria were mainly short rods or cocci. They were rarely attached at right angles to the algal cells and none were as large as the bacteria seen on the Synedra cells.

b) Addition of lake water to Diatoma elongatum culture

After 2 days, the culture was heavily bacterized but the diatoms were healthy and no bacteria could be seen attached to them. After 14 days incubation, only about three-quarters of the algae appeared to be healthy. Although the Chrysophycean had colonized many of the diatoms, no epiphytic bacteria, attached perpendicularly, could be seen. At the ends of some of the algal cells, clumps of bacteria were visible (Fig. 8.7).

c) Enrichment for Caulobacter

Although samples of the enrichments were examined at frequent intervals, only a few stalked bacteria were seen after 12 days incubation. These were attached to chains



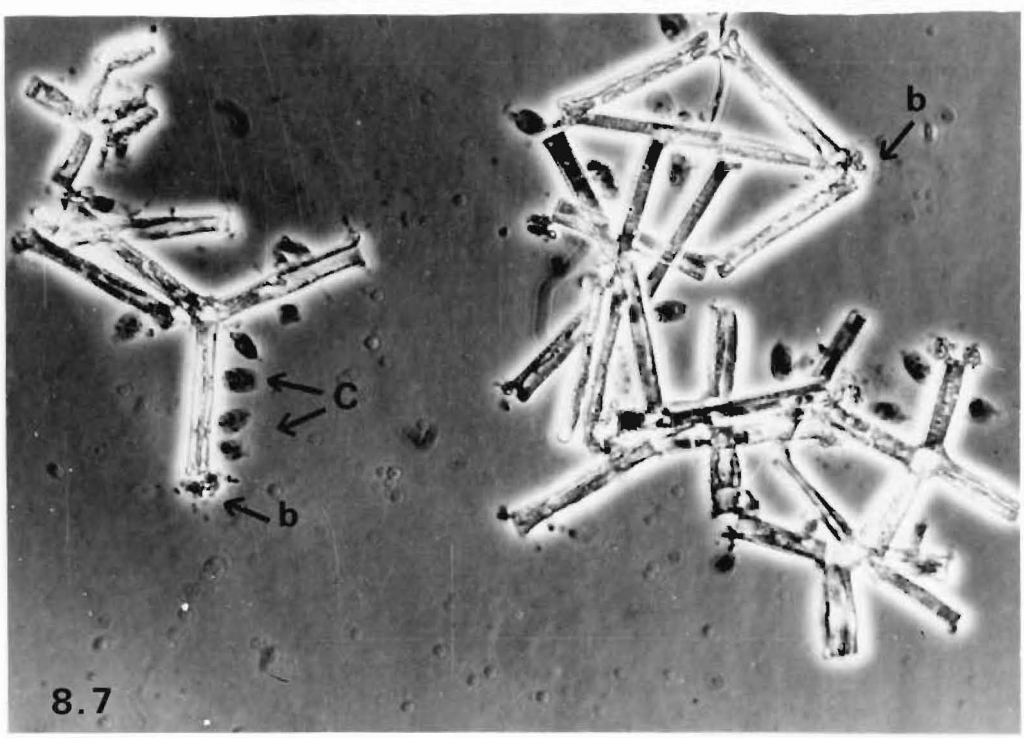


FIG. 8.6 Synedra ulna cell showing epiphytic bacteria; x 600.

FIG. 8.7 Diatoma elongatum in culture, to which lake water was added, showing colonization of cells by Chrysophycean (C) and bacteria (b); x 600.

of bacteria and they failed to grow on the agar medium.

Comparison of epiphytic bacteria and cultured bacteria

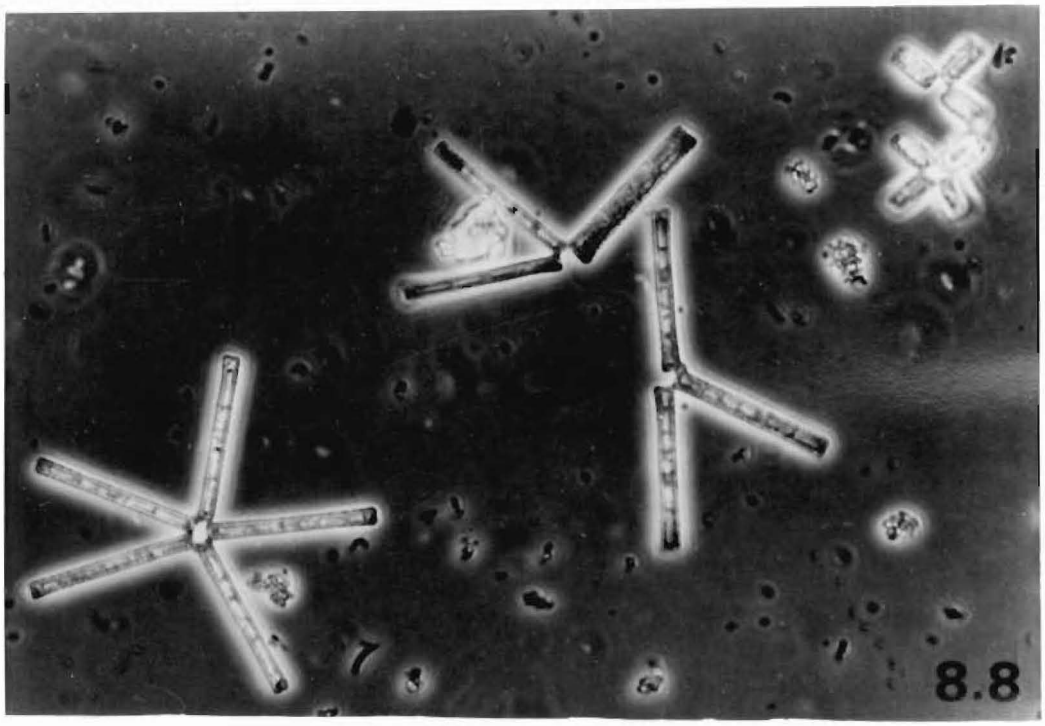
a) Uptake of radioactive compounds

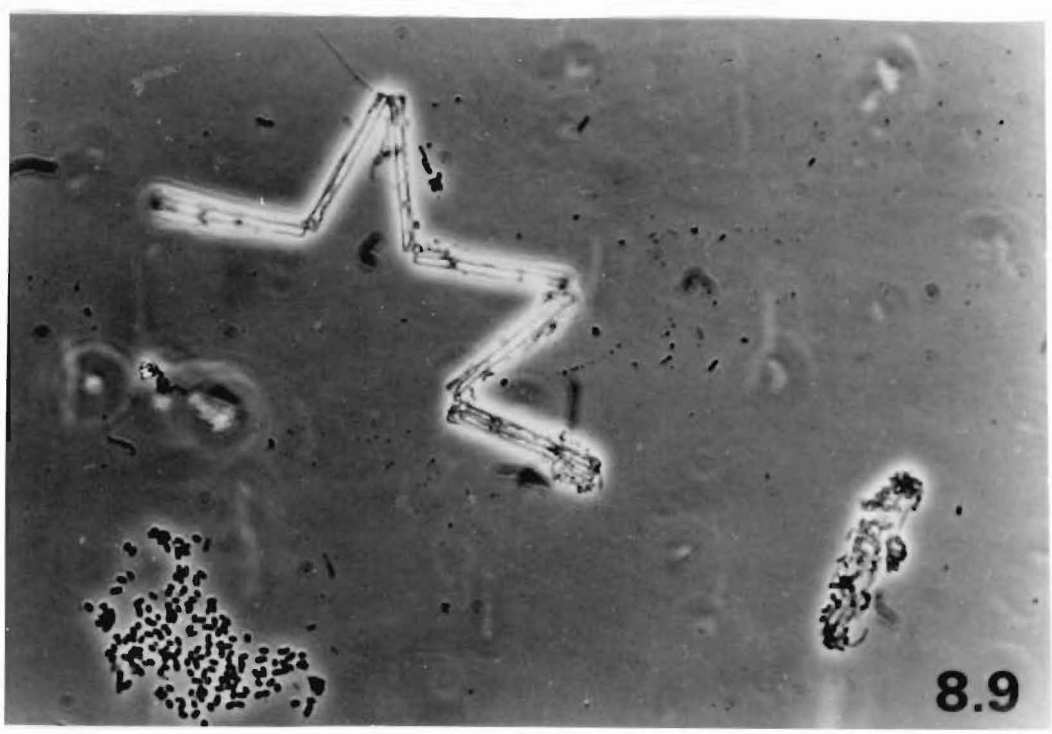
The sample collected in March, 1971, included Melosira with bacterial epiphytes. No uptake of ^{3}H-glucose or ^{3}H-thymidine by these bacteria was detected by autoradiography. In the second sample, collected in January, 1972, there were many bacteria on Asterionella and Fragilaria cells, but none became labelled with ^{3}H-glucose.

b) Attachment of bacteria isolated from Lake Grasmere to Diatoma elongatum in culture

(i) Healthy and moribund algal cells from each tube were examined after incubation for 15 to 60 min and 48 h, but in no case were any bacteria seen attached to algal cells.

(ii) In the longer experiment with Diatoma and Vibrio extorquens, examination after 1 to 6 days showed that there were almost no bacteria attached to healthy or dead algae (Fig. 8.8). Two moribund algal cells were seen which may possibly have had bacteria attached to them. After 18 days there was a large bacterial population in the tube and all the algae looked unhealthy, but many cells were still in colonies. The majority of the algae were still bacteria-free but some of the dead cells had one or two bacteria on them. Also, one dead cell was seen which was covered in bacteria and, occasionally, there were fairly





FIGS. 8.8 and 8.9 Cells of Diatoma elongatum in culture, showing incidence of attachment by Vibrio extorquens.

Fig. 8.8 - incubated for 4 days; no bacteria attached; x 620. Fig. 8.9 - incubated for 18 days; many bacteria attached; x 580.

healthy cells with bacteria around them (Fig. 8.9).

(iii) Numbers of bacteria did not increase so much in the tap water and more algae had bacteria attached in the algal medium than in the tap water. However, in general, attachment of bacteria to algal cells was rare and confined to moribund or dead cells. Even then, not all moribund cells were colonized. More cells were colonized when they had been incubated for 4 days than after incubation for 18 h. There was little difference in the behaviour of the bacterial cultures except for the Cytophaga isolate, which grew in clumps some of which were seen at the ends of moribund cells. None of these bacteria was seen attached at right angles to the cells.

c) Bacterial population of bacterized algal cultures

Examination of old cultures of the longer and shorter taxa of Diatoma elongatum showed that the longer taxon, which was not obtained bacteria-free, had an epiphytic bacterial population but this was confined to some of the dead cells. These bacteria grew in diffuse clumps and at the corners of the cells (Fig. 8.10). In the old cultures of the smaller taxon, which was finally obtained in a 'nearly bacteria-free' state, none of the algae had an epiphytic flora even in cultures which contained many bacteria.

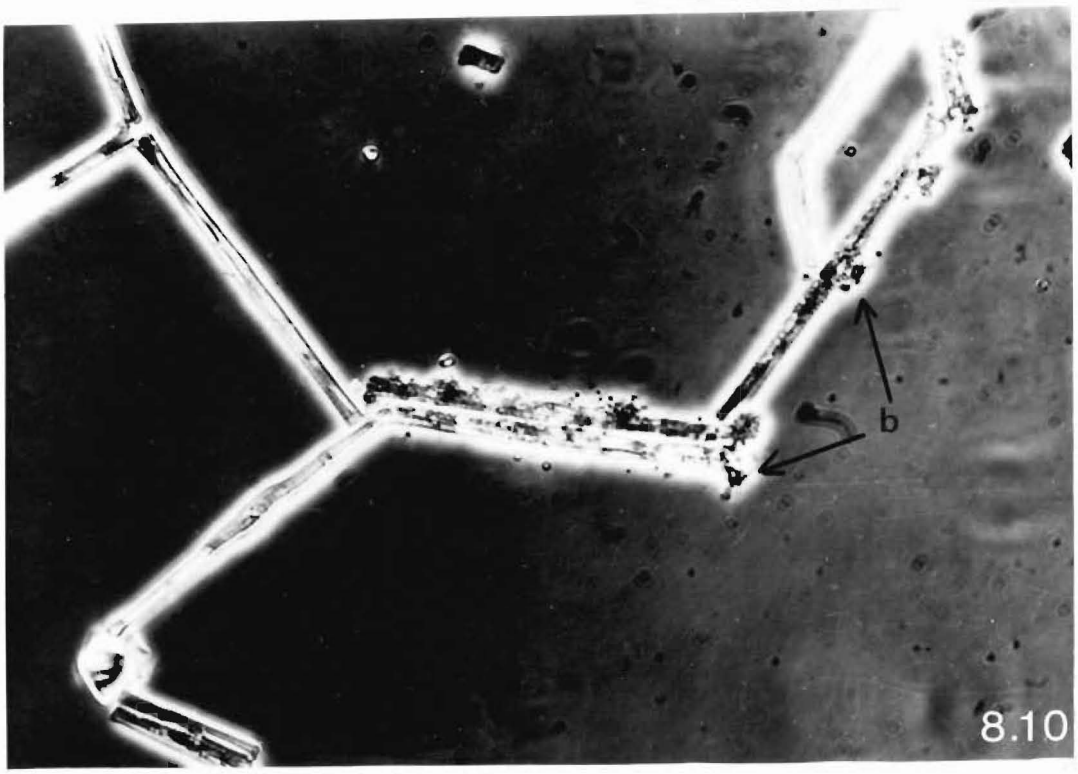
When a loopful of culture of the longer taxon was added to a culture of 'nearly bacteria-free' shorter taxon, the shorter taxon remained very healthy and no dead cells could be found after 7 days incubation. The culture was still healthy when left standing in the laboratory for a further week, so another loopful of the longer taxon culture was added to the test culture. After 14 days, the culture was moribund with no colonies visible. Some of the algae had bacteria on them but they were rarely at right angles (Fig. 8.11).

Possibility of formation of toxins by Diatoma elongatum

The possibility that Diatoma elongatum produced a toxin which prevented the bacteria isolated from Lake Grasmere from attaching to the diatom in culture was tested by assessing the rate of growth of bacteria on Millipore filters, part of which had algal cells beneath them. The fluorescent pseudomonad did not grow on the quarter which had algal cells between the filter and agar. No difference in growth between the control and the algal quarter could be distinguished with the other three isolates. However, Vibrio extorquens was conspicuous in that it grew far more vigorously than the other isolates on both quarters.

8.3 DISCUSSION

Direct observations of field samples indicated that several kinds of bacteria were capable of attaching to living algae. These bacteria differed morphologically and in their ability to colonize healthy cells. The presence of epiphytic



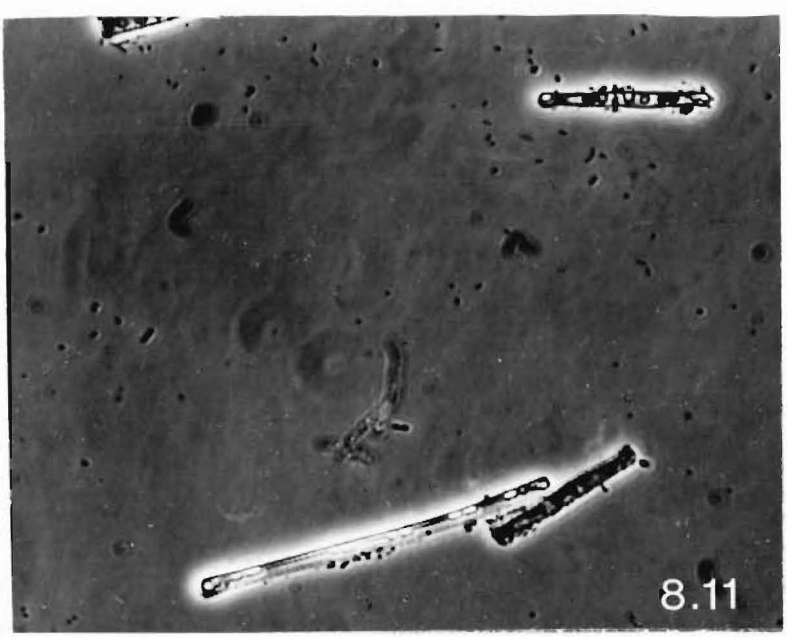


FIG. 8.10 Cells of 'longer taxon' of Diatoma elongatum after culture for 5 weeks, showing bacterial colonization (b); x 580.

FIG. 8.11 Colonization of 'shorter taxon' of Diatoma elongatum after inoculation with bacteria from culture of 'longer taxon'; incubated for 28 days; x 580.

bacteria during the early stages of an algal bloom was influenced by environmental factors as the same species of alga was not always colonized.

The observations made between November, 1970, and April, 1971, suggested that healthy algae - both diatoms and green algae, which might be expected to be more susceptible to colonization because of their mucilaginous sheaths - very rarely had a bacterial flora. This confirmed the reports of Steemann Nielsen (1955), Saunders (1959), Oppenheimer and Vance (1960), Overbeck and Babenzien (1964), Droop and Elson (1966)and Sieburth (1968). Furthermore, most moribund and dead diatoms were not colonized in the water by bacteria. However, Melosira granulata var. angustissima did have a bacterial flora which was not seen on other kinds of algae present in the same samples. Melosira is a filamentous diatom. Each filament is made up of about 14 cells and these cells may be more susceptible to colonization than the single cells of Diatoma, Asterionella and Cyclotella. These bacteria were only found when algal numbers had dropped and the remaining cells were presumably less healthy. Droop and Elson (1966) also found that the numbers of epiphytic bacteria on a marine diatom only began to mount when the algal cells were becoming moribund and numbers were declining. There was, however no evidence that the bacteria attached to Melosira were involved in decomposition of the algae. Not all moribund or dead filaments had a bacterial flora. No uptake of ^{3}H-glucose or ^{3}H-thymidine was detected and plate counts increased only

slightly at the end of the Melosira bloom so that few if any of the these bacteria were being cultured. The lack of any marked increase in plate counts also indicated that the epiphytic bacteria were not bringing about any release of nutrients which could be utilized by the kinds of bacteria which were cultured. Similar results were obtained by Taylor (1949) at the end of an Asterionella bloom when the algae had a conspicuous epiphytic flora.

The bacteria on Melosira were not identified. It is possible that they were dead bacteria adsorbed to the algal cells, but the work of Meadows (1971) makes this suggestion unlikely. Their epiphytic habit indicated they might be a species of Caulobacter, but no stalks were seen.

The epiphytic bacteria, which were common on Asterionella and Fragilaria in the summer of 1971/1972, differed from those on Melosira. They were all unicellular and were found on healthy as well as moribund algae. However, as with the epiphytes of Melosira, no uptake of ^{3}H-glucose by the epiphytes of Asterionella or Fragilaria was detected by autoradiography, although this experiment was not conclusive as the sample was kept for 24 h before labelling. The epiphytes of Asterionella were also not cultured, even when living Diatoma elongatum cells were included in one medium in case the bacteria were obligate epiphytes. It is possible that the nutrient concentration in this medium was inhibitory or this alga was resistant to attachment.

The epiphytic flora of Asterionella was similar to that described by Taylor (1949). He reported that the appearance of the bacteria coincided with the maximum growth of the algae. In late 1971, samples were only examined for bacteria

when the numbers of Asterionella were beginning to decline. Epiphytic bacteria were present. At the beginning of another increase in numbers of Asterionella in March, 1972, epiphytic bacteria were very rare. These results do not contravert the possibility that epiphytic bacteria are confined to algae in a declining population. However, in December, 1971, many apparently healthy cells were colonized. As Asterionella was not colonized at any stage of the small peak in the summer of 1970/1971, it is possible that the environmental conditions favoured colonization throughout the bloom in late 1971. Asterionella does not usually have a peak in the summer in Lake Grasmere.

When epiphytic bacteria were common on Asterionella, there was a morphologically different bacterial population on Synedra ulna, which was found in the littoral water samples. Attempts to culture these bacteria also failed.

Thus, there were several kinds of epiphytic bacteria present occasionally, whose mode of nutrition was not clear. These bacteria did not appear to be typical Caulobacter spp. as enrichment for Caulobacter was unsuccessful and no stalks were seen. Similar epiphytic bacteria were not seen on algal fragments in the mud, but some of the living diatoms in the mud were colonized. This is in contrast to the observations of Oppenheimer and Vance (1960) who found that only when algae died were they attacked by bacteria. However, in the present study the mud samples included fragments of algae, but not many algae which had recently died. The absence of bacteria may have been because decomposition of these fragments was complete. However, from the evidence

available, it would appear that the epiphytic bacteria in Lake Grasmere were more often associated with living algae than dead ones.

One characteristic feature of the epiphytic bacteria was that they were all attached at right angles to the algal cells. This mode of attachment was also noted by Saunders (1959), Oppenheimer and Vance (1960) and Sieburth (1968).

In the experiments testing the attachment of bacteria isolated from Lake Grasmere, and bacteria in bacterized algal cultures, to Diatoma elongatum in culture, bacteria were rarely attached at right angles to the diatom cells. Live diatoms were not colonized and colonization of moribund and dead diatoms was slow and confined to only some of the dead cells. A low level of nutrients did not encourage attachment by bacteria as noted by Ruschke (1968). This confirmed that the bacteria seen attached to algae in the field samples were not similar to those cultured.

The small experiment on the inhibitory effects of a large number of diatom cells suggested that the fluorescent pseudomonad might be inhibited by diatoms. However, as the other bacteria were not inhibited the lack of attachment of the bacteria was not due to the formation of a non-specific toxin by the algae.

Ecological significance of epiphytic bacteria

One of the main points apparent from the seasonal study of bacteria and algae described in Chapter 7 was the lack of a relationship between algae and the heterotrophic bacteria

which were cultured. This led to the questions of how and where algae were being broken down. The direct observations reported in this chapter indicated that on occasions there were bacteria attached to algae for example, Asterionella, and that on some algae, such as Melosira, they were confined to some of the moribund cells. However, these bacteria were not cultured and were not present on all moribund algae. It therefore seemed unlikely that they had a major role in the decomposition of algae. The absence of such bacteria on algae in the mud also suggested that they might be dependent on living algae for nutrients.

In studies when numbers of bacteria have been estimated by the direct and indirect methods, direct counts almost invariably have given larger counts (Snow and Fred, 1926; Rodina and Kuz'mitskaya, 1963; Drabkova, 1965; Jannasch, 1965). Technical reasons for such discrepancies have not been put forward and the possibility that certain kinds of bacteria are not included in the plate counts has been considered (Jannasch, 1965; Goldman et al., 1968). In the present study direct counts were never very successful, but the difference between the direct count and plate count was larger in December, 1971, when there were many bacteria on Asterionella (Table 3.1). In this case the increased discrepancy may well have been due to the presence of bacteria able to colonize the algae but not cultured. As the sample was macerated to break up clumps, there is no indication of the proportion of these bacteria normally free in the water. However, it is possible that such bacteria may often account for some of the difference between direct counts and plate counts of bacteria. If this is so it would

be important to determine their mode of nutrition as the data from this study would suggest that many of these bacteria are not involved in heterotrophic breakdown of compounds. Thus, a direct count may sometimes not give a meaningful indication of the amount of decomposition occurring in a lake.

In interpreting data from a study of this kind, there is the problem of distinguishing between effects which are found universally and those which are due to some characteristic of the habitat being studied. Thus, the lack of epiphytic bacteria on many healthy algae (Steemann Nielsen, 1955 and others), an increase in numbers of epiphytic bacteria on some moribund algae (Droop and Elson, 1966), even epiphytic bacteria being occasionally on live algae but not on dead (Overbeck and Babenzien, 1964), were all observed at some time in this study. However, the bacterial colonization of dead algae observed by Oppenheimer and Vance (1960) in samples from marine sediments, with many of the bacteria attached perpendicularly, was not confirmed in this study.

In the present study of a freshwater environment only epiphytic bacteria, which could not be cultured and appeared mainly on living algae, were always found attached perpendicularly, while bacterial colonization by the kinds of bacteria routinely cultured was slow and rarely at right angles to the algal cell. As Oppenheimer and Vance (1960) were examining marine sediments, the ionic effects of sea water may have influenced the mode of attachment of all kinds of bacteria.

As algae were not rapidly decomposed by bacteria in Lake Grasmere, these observations raised the question of the role of attachment of bacteria, and the angle of attachment, in the breakdown of algae. If bacterial attachment is essential to initiate breakdown, the factors controlling this attachment may be of major importance in determining the rate of recycling of nutrients in Lake Grasmere. However, Golterman (1964) considers that autolysis is an essential step in the mineralization of algae. In Lake Grasmere there was only a small response to declining algal numbers suggesting that little spontaneous release of nutrients was occurring. Alternatively if some combination of nutrients was required before attachment could take place these might also be necessary before nutrients, released from algae by autolysis, could be utilized.

Thus, little bacterial colonization of living algae would appear to be universal, apart from certain special epiphytic bacteria. However, the apparent resistance of dead algae to bacterial attachment would appear to be characteristic of Lake Grasmere. The factors controlling this attachment may be basic to an understanding of the reasons for the small bacterial population in the water of Lake Grasmere.

Further studies on the role of the epiphytic bacteria and the factors controlling their presence and attachment, on the attachment of bacteria routinely cultured to algae, and the mechanisms of breakdown of algae <u>in situ</u> should help to solve this problem. Apart from these questions concerning the reasons for the limited bacterial colonization of algae and the low numbers of bacteria in the water, there

is also the problem of where most bacterial decomposition of plankton occurs. This will be considered further in the next chapter in which bacterial activity in the mud is discussed.

8.4 SUMMARY

1) Direct observations of algae from the lake showed that the majority were free of any epiphytic bacteria. However, some moribund cells of Melosira granulata var. angustissima had bacteria attached to them and, during the summer of 1971/1972, healthy and moribund cells of algae, including Asterionella formosa, were heavily colonized by bacteria. Attachment of the bacteria was always at right angles to the algal cell.

2) Attempts to culture the epiphytic bacteria on a range of media failed and the bacteria were not identified.

3) The kinds of bacteria, which were isolated successfully from the lake, did not attack healthy cells of Diatoma elongatum in culture. The majority of the moribund cells were also free of bacteria and few of those that were colonized had bacteria attached perpendicularly.

CHAPTER 9

THE IMPORTANCE OF BACTERIA IN THE MUD IN THE RECYCLING OF NUTRIENTS

If bacteria are the main agents in the mineralization of nutrients from plankton and there is little bacterial response in the water to declining numbers of plankton, the most likely site of bacterial activity is the mud (Taylor, 1949). Decomposition of organic matter in the sediment may be one of the main sources of new organic matter in the water column if there is little introduction of nutrients from outside sources (Seki, Skelding and Parsons, 1968). However, although a slow recycling of nutrients in mud may be related to low productivity in lake water (Henrici and McCoy, 1938; Carpenter, 1939; Cooper, Murray and Kleerekoper, 1953), the numbers of bacteria in a sediment are not always directly related to the productivity of the lake (Potter and Baker, 1956; Hayes and Anthony, 1959). Potter and Baker found a much smaller number of bacteria in the mud from an open water site of a eutrophic lake than in the mud from a similar but deeper area of an oligotrophic lake. Bacteria isolated from the mud of a eutrophic lake proved to be less metabolically-active than bacteria from the water (Strzelczyk and Mielczarek, 1971). The rate of decomposition in the lake water and the amount of organic matter and minerals being brought into the lake through inlets will both determine to what extent nutrient levels in the water

are dependent on bacterial activity in the mud.

Fluctuations in the numbers of bacteria in the mud from one sampling date to the next have been recorded, but, in most cases, no consistent response to a particular factor has been detected (Reuszer, 1933; Henrici and McCoy, 1938; Hayes and Anthony, 1959; Jones, 1971; Nedwell and Floodgate, 1971). However, Williams and McCoy (1935) did not find any significant difference in the numbers of bacteria in mud sampled in summer and winter. Cooper, Murray and Kleerekoper (1953) found that numbers of bacteria in lake mud increased in several areas of the lake at the same times, but they could not correlate these increases with any specific parameter. Increases in the bacterial population of Lake Erie mud were observed to follow periods of phytoplankton maxima and those periods of high turbidity of lake water which were caused by river discharge (Weeks, 1944). Numbers of bacteria in the sediment were not correlated with the organic content of the sediment (Anthony and Hayes, 1964).

Previous studies of the bacteria of mud and water have shown that the benthic microflora is distinct from the water population. A larger proportion of Gram-positive bacteria was found in the mud, and spore-forming bacteria were usually present (Potter, 1964; Strzelczyk, Antczak and Kuchcińska, 1971). Reuszer (1933) observed that the surface layer of marine mud supported more kinds of bacteria than the lower regions. The bacterial population of the surface mud was also larger.

Henrici and McCoy (1938) and Weeks (1944) also found larger bacterial populations in the surface layers of some

of their mud samples but Hayes and Anthony (1959) were unable to find significantly larger populations in the top layers of lake sediments compared with the population in mud about 5 cm below the surface.

This chapter describes the size and seasonal fluctuations of the bacterial population in the mud and relates these to the bacterial and plankton populations of the water. The kinds of bacteria in the mud are also compared with the water population and the activity of the bacterial population in the mud is discussed.

9.1 METHODS

Mud was sampled from beneath open water at monthly intervals between May, 1970, and March, 1971. Open water was sampled from 3 m at the same times. In April and June, 1969, mud was sampled within the weed bed, and samples of water over weed were also included. Details of sampling dates are given in Appendix 9. The numbers of bacteria in the mud and water samples were determined by the pour plate method as described on pp. 22, 23 and 32.

The volume of the dominant diatoms in the water samples was determined as described on p.213 between November, 1970, and March, 1971. Apart from four counts done by the author, data on the zooplankton populations for the period from May, 1970, to March, 1971, were obtained from Dr V.M. Stout.

In April, 1971, mud beneath open water was sampled and the number of bacteria in the brown surface layer of the mud, which was about 0.5 cm thick, was compared with that in the grey mud, 3 to 5 cm beneath the surface. Because

the brown layer was not extensive, instead of the usual 5 g samples, 0.6 to 0.9 g of mud was sampled with a pipette and plated out.

The kinds of bacteria in the surface layer of mud and in lake water were studied in July, 1971. Bacteria were also characterized in April and June, 1969.

The metabolic activity of the bacteria in the mud sampled in July, 1971, was examined by labelling them with ^{3}H-glucose or ^{3}H-thymidine and preparing autoradiograms as described on p. 81. A single autoradiogram was prepared for each treatment.

9.2 RESULTS

Size of the benthic bacterial population and fluctuations in this population compared with the fluctuations in the bacterial and plankton population of the water

From May, 1970, to March, 1971, the numbers of bacteria in the mud varied from $0.45 \times 10^{6} \pm 0.09 \times 10^{6}$ to $4.45 \times 10^{6} \pm 0.96 \times 10^{6}$ per g dry wt of mud. The bacterial population of the mud sampled from among the weed bed in 1969 was within this range, viz. April - $2.19 \times 10^{6} \pm 1.02 \times 10^{6}$; June - $2.04 \times 10^{6} \pm 0.28 \times 10^{6}$ (per g dry wt of mud). Samples of surface and subsurface mud were plated out in April, 1971, to compare the numbers of bacteria in each fraction. As small samples were used, estimation of their dry weight from replicate samples was not easy and the results may not have been comparable with those reported above. However, this experiment did show that twice as many bacteria were plated

out from the subsurface sample as from the surface mud, viz. surface - $1.6 \times 10^6 \pm 0.17 \times 10^6$; subsurface - $3.18 \times 10^6 \pm 1.62 \times 10^6$ (per g dry wt mud).

In Fig. 9.1, the seasonal fluctuations in the numbers of bacteria in the mud are compared with the fluctuations in the bacterial and plankton populations of the open water. The bacterial populations of the mud and open water fluctuated inversely except in December and January. The increase in numbers of bacteria in the mud in October followed the heavy rains of August and September, when a large amount of silt and attached bacteria was brought into the lake. The winter peak in the benthic bacterial population in July and August followed the period of decreased growth and death of Elodea which occurred in the autumn months.

At some times of the year the benthic bacteria appeared to respond to fluctuations in the plankton populations in lake water (Fig. 9.1). The peak in January, 1971, followed the October bloom of rotifers and diatoms, but it also coincided with an increase in the lake bacterial and plankton populations. No increase in the numbers of bacteria in the mud was detected after the summer bloom of Melosira and Cyclotella and some rotifers, although sampling was continued for a month after plankton numbers had decreased. No data on the phytoplankton was available for the period of May to October. There was a peak in the rotifer population in early July before the increase in the numbers of bacteria in the mud occurred in late July.

Kinds of bacteria in mud and water

The kinds of bacteria in mud and lake water over the

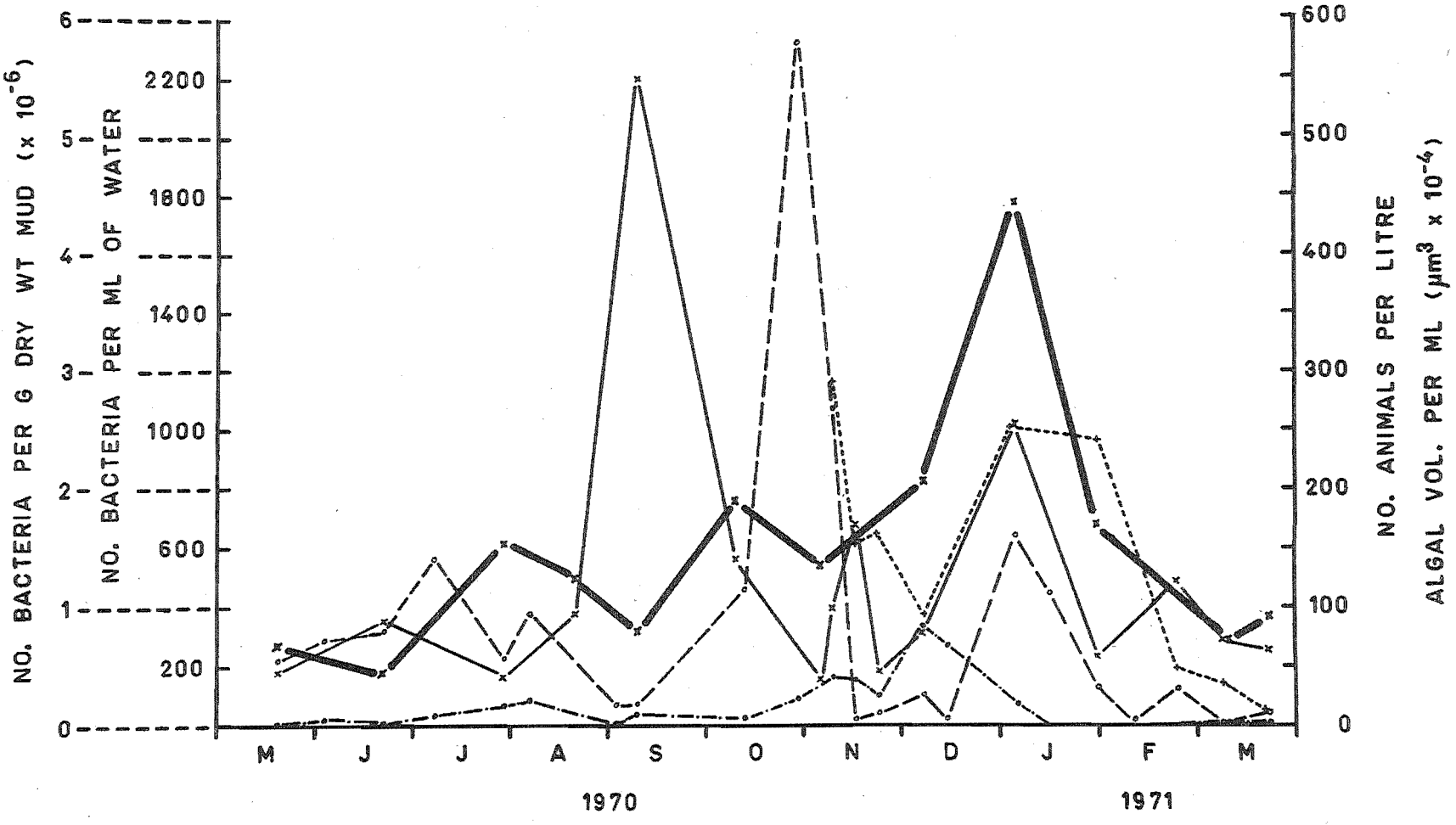


FIG. 9.1 Seasonal fluctuations in plankton and bacterial populations of open water and bacterial population of mud. Bacteria from mud —×— , bacteria from open water —×— , diatoms ----•---- , rotifers ——∘—— , Cladocera —·—·—·—.

mud were studied on three occasions (Table 9.1). In 1969, mud was sampled from among the weed and samples were a mixture of surface and subsurface mud to a depth of about 8 cm. There were varying degrees of similarity between the kinds of bacteria in the mud and water samples. In April, 1969, the mud and water over weed samples had similar percentages of Alcaligenes/Achromobacter, flavobacteria and Cytophaga, but the mud had larger proportions of coryneforms and Bacillus. The mud sampled in June had a bacterial flora more distinct from the water sampled at the same time. Bacillus made up a major part of the benthic microflora and the only groups of bacteria which were present in similar percentages in both samples were coryneforms and pseudomonads. The benthic bacteria characterized in July, 1971, were from surface mud sampled beneath the open water. These bacteria were far more similar to the water bacteria than those in the samples collected in 1969 (Table 9.1). No Bacillus was found in the 1971 mud sample. The percentages of pigmented bacteria varied within the three sets of mud and water samples (Table 9.2).

Metabolic activity of benthic bacteria

Labelling surface mud sampled in July, 1971, with ^{3}H-glucose and ^{3}H-thymidine indicated that many of the benthic bacteria were not metabolizing at this time. Only 47% of the bacteria labelled with ^{3}H-glucose, and 27% of those labelled with ^{3}H-thymidine, accumulated enough label to form an autoradiogram.

TABLE 9.1 Kinds of bacteria in mud and lake water

Date sampled	Sample	No. characterized	Percentage distribution												
			Alcaligenes/ Achromobacter	pseudomonads	flavobacteria	Cytophaga	Vibrio extorquens	Enterobacteriaceae	Aeromonas/ Vibrio	Acinetobacter	Chromobacterium	coryneforms	Micrococcaceae	Bacillus
15/ 4/69	mud	72	8	10	10	20		+				32		17
15/ 4/69	water	67	12	25	18	21	6	+				8	6	
17/ 6/69	mud	51	+	10	+	+	+	+	+			16	+	58
17/ 6/69	water	72	+	8	21	8	24	+				22	13	
13/ 7/71	mud	45	9	35	25				+		7	20		
13/ 7/ 71	water	44	+	28	22		8		+		+	33		+

+ ≤ 5%

TABLE 9.2 Percentage of chromogens in samples of mud and lake water

Date sampled	Sample	No. characterized	Chromogens (%)
15/4/69	mud	72	36
15/4/69	water	67	49
17/6/69	mud	51	16
17/6/69	water	72	68
13/7/71	mud	45	40
13/7/71	water	44	61

9.3 DISCUSSION

Sampling technique

In this study samples of mud were required which would indicate the rate at which organic matter was being decomposed and nutrients recycled. Mud was sampled with an Ekman dredge and, for the counts of bacteria reported in Fig. 9.1, was a mixture of the thin, brown surface layer and the grey mud from up to 8 cm below the surface. Decreasing numbers of bacteria in the top few cm of some mud samples have been reported (Reuszer, 1933; Henrici and McCoy, 1938), but Hayes and Anthony (1959) found no significant decline in counts down to about 5 cm below the surface.

Below this depth there was a gradual decrease in numbers of bacteria. The surface sample analyzed here did not have a larger population than that of the mud from a lower region (p.278).

It is therfore unlikely that the differences in numbers of bacteria between samples, which were of mud within the top 5 to 8 cm, were due primarily to the sampling technique.

Size of the benthic bacterial population and fluctuations in this population compared with the fluctuations in the bacterial and plankton populations of the water

Previous studies, in which the bacteria in 'top' mud from deep water stations have been estimated per gram dry weight of mud, have found average populations ranging from 8.6×10^3 in oligotrophic Crystal Lake (Carpenter, 1939) to 0.074×10^6 - 2.079×10^6 for 16 lakes of varying nutrient concentrations (Hayes and Anthony, 1959) to 5.2×10^6 for eutrophic Lake Mendota (Williams and McCoy, 1935) to 2.628×10^6 - 7.731×10^6 in Lake Erie (Weeks, 1944). The bacterial population of the mud of Lake Grasmere fluctuated within this range but tended to be higher than those reported by Hayes and Anthony (1959). However, as an indication of the productivity of a lake, numbers of bacteria by themselves are not easy to interpret as they will be influenced by the nature of the organic matter deposited on to the mud. Potter and Baker (1956) found that the mud of Roger's Lake, classified as eutrophic, had a much smaller bacterial population than oligotrophic Flathead Lake. Hayes and Anthony (1959) compared the numbers of bacteria in the surface sediment of 16 lakes with parameters of the water, such as methyl orange

alkalinity and conductivity. Three of the lakes had too few bacteria, and one had more bacteria, in the sediment than was expected from the chemical properties of the water. In a eutrophic lake where much decomposition occurs in the lake water, only less readily assimilated compounds will reach the mud. Strzelczyk and Mielczarek (1971) found that benthic bacteria from a eutrophic lake were less metabolically-active than the water bacteria. The numbers of bacteria in the mud of such a lake may thus not be very different from those of a nutrient-poor lake where much of the organic matter is deposited unchanged. In the former case decomposition may be limited because of the complexity of the compounds deposited; in the latter, recently sedimented matter may be utilized only slowly because essential minerals are lacking.

An analysis of the seasonal fluctuations in numbers of bacteria in the mud is more informative as the response of the benthic bacteria to the introduction of organic matter indicates how important these bacteria are in the recycling of nutrients. In previous studies, seasonal fluctuations in the numbers of benthic bacteria have been recorded, but in most cases these have not formed any logical pattern (Reuszer, 1933; Weeks, 1944; Cooper, Murray and Kleerekoper, 1953; Hayes and Anthony, 1959; Jones, 1971; Nedwell and Floodgate, 1971).

In Lake Grasmere the inverse fluctuation of the bacterial populations of the mud and open water suggested that the same factors were influencing both the benthic and water bacterial populations but with a lag period before any res-

ponse by the benthic bacteria was apparent (Fig. 9.1). Sedimentation of weed fragments and attached bacteria may have been one cause of the increased bacterial population of the mud in July and August. However, this peak could have been related to the increased zooplankton population in July or to a phytoplankton peak, but data were not available on the phytoplankton during this period. After the inflow of silt in September with the large increase in the bacterial population of the water, numbers of bacteria in the mud increased. This suggested that the benthic bacteria were responding to similar sources of nutrients once these had sedimented on to the mud. Alternatively the benthic bacterial population may have increased because of direct sedimentation of bacteria. A similar increase was noted by Weeks (1944).

Although the increase in numbers of bacteria in the mud in January was possibly in response to the blooms of diatoms and rotifers in October, a similar response was not detected after the summer phytoplankton maximum. The peak in numbers of bacteria in January coincided with an increase in the numbers of bacteria, algae and zooplankton in the water. The temperature of the open water had risen to 16.7°C and, on 14 December, phosphate was at a measurable level of 0.003 mg/l in the open water for the first time for six months. Phosphorus is one element essential in bacterial growth (Johannes, 1968). It is possible that the presence of phosphate and the warmer water temperature triggered off bacterial decompositon of the large numbers of diatoms and rotifers which sedimented on to the mud

during November. At the same time there was an increase in the bacterial and plankton populations in the lake water which may have been due partly to the phosphate in the water in December and partly to the release of nutrients from the increased activity of bacteria in the mud. After the summer bloom of algae and zooplankton, the lack of a similar response may have been because nutrients such as phosphate were limiting (Fig. 4.2).

The uppermost layer of the mud consisted of a layer of recently deposited organic matter. Hayes and Anthony (1959) anticipated that decomposition would occur more rapidly in this region than in the subsurface mud, but they were unable to find a significantly larger bacterial population in the top layer of the sediment (mean depth 0.25 cm) compared with a second slice of mean depth 0.75 cm in 40 cores from 12 lakes. They concluded that if a high-count surface layer existed it must be extremely thin ie. 0.001 cm. Earlier workers had found larger bacterial populations in some surface mud samples (Reuszer, 1933; Henrici and McCoy, 1938; Weeks, 1944). In Lake Grasmere, the surface mud was distinct from the lower regions in its yellow-brown colour and was at least 0.5 cm deep. In April, 1971, there was a smaller bacterial population in the surface layer. The experiments discussed in Chapter 7 indicated that little bacterial decomposition was occurring in the lake water. The counts of the surface mud suggested that this decomposition continued only slowly in the recently sedimented layer. In the absence of essential minerals further decomposition may not take place until the organic matter has

been broken down, perhaps through ingestion by animals.

Kinds of bacteria in mud and lake water

The present study confirmed the observations of Potter (1964) and Strzelczyk, Antczak and Kuchcińska (1971) that the bacterial population of mud differed from that of water. However, this difference was greater between some of the mud and water samples than others (Tables 9.1 and 9.2).

The bacterial population of the surface mud sample was most similar to the water population. The lack of a much larger population of bacteria in the surface layer of mud compared to the subsurface mud was discussed above. It is likely that the similarity of the kinds of bacteria in the surface mud and water is directly related to the similarity in their nutrient source. With the slow rate of decomposition in the surface mud the organic matter will not be very different from that in the lake water.

A gradual change in kinds of bacteria would be expected with increase in depth of mud. In this study the two samples which were more similar to the water population at the time of sampling had the largest proportions of pigmented bacteria (Table 9.2). It can be concluded that in Lake Grasmere pigmented bacteria will usually only be found in the regions of the mud where decomposition is at the early stages. In previous studies, the range of chromogens in mud samples has been wide. Potter (1964) reported percentages varying from 14% to 39%, while Strzelczyk and Mielczarek (1971) studied the metabolic activity of 58 benthic bacteria of which 45 were chromogens. However, Hayes and

Anthony (1959) found pigmented bacteria so rarely in mud samples that they concluded that these bacteria were not part of the indigenous bacterial population. The results obtained here suggest that the conclusion of Hayes and Anthony (1959) is not strictly justified as these bacteria may play an essential part in the early stages of decomposition in the mud although they may soon be displaced by other bacteria when some breakdown of organic matter has occurred. The oxygen levels in the mud may also affect their survival.

Metabolic activity of benthic bacteria

The labelling of the benthic bacteria with ^{3}H-glucose and ^{3}H-thymidine indicated that much of the benthic population was not metabolizing in the winter at least. In addition, examination of mud smears on several occasions showed that the numbers of bacteria were very small compared to the amount of detritus and algal fragments in the mud. This did not suggest a rapid bacterial decomposition of these particles.

The low numbers of bacteria in the mud visible by direct examination and the often limited response of the benthic bacteria to added nutrients, both confirmed that bacterial activity in the mud of Lake Grasmere could be limiting the release of nutrients and be a cause of the small numbers of bacteria in the water. However, there were still sufficient nutrients to support widespread growth of Elodea and phytoplankton although bacterial decomposition of these in the water and mud was not apparently rapid. The two samples of mud from among the weed did not have sufficiently

large populations to suggest that far more decomposition was occurring in the mud among the weed than beneath the open water.

This suggested that nutrients required for primary production were available but were not being released continually from the mud by bacterial action. As bacterial activity throughout the lake, except possibly on Elodea, was not extensive it was of interest to study some of the groups of bacteria which were found in the water to find out whether they were associated with a particular habitat or kind of substrate. In the following two chapters the ecological niches of Cytophaga and Vibrio extorquens are considered.

9.4 SUMMARY

1) The mean numbers of viable bacteria in mud sampled from up to 5-8 cm below the surface varied from 0.45×10^6 to 4.45×10^6 per g dry wt of mud. Numbers of bacteria in a sample of the surface layer of mud were only half those in a sample of mud from up to 5 cm below the surface sampled at the same time.

2) Direct observations of mud samples showed that numbers of bacteria were low compared to the amount of detritus and algal fragments present.

3) Increases in numbers of viable bacteria in the mud were observed after heavy rain when much silt was brought into the lake, and following a peak in numbers of both rotifers and diatoms. However, there was not usually

an increase in the numbers of benthic bacteria at the end of a bloom of zooplankton or phytoplankton.

4) Bacteria in mud sampled in winter, 1971, were labelled with ^{3}H-glucose and ^{3}H-thymidine. Forty-five percent of the bacteria labelled with ^{3}H-glucose, and 27% of those labelled with ^{3}H-thymidine, formed an autoradiogram.

5) The kinds of bacteria in mud from up to 8 cm below the surface differed from the water population in the larger proportion of Gram-positive bacteria. However, a surface mud sample had a bacterial population very similar to the water population at that time.

CHAPTER 10

THE ECOLOGY OF CYTOPHAGAS ISOLATED FROM LAKE GRASMERE AND ITS INLETS

The genus *Cytophaga*, as described by Winogradsky (1929), was restricted to a group of non-fruiting, cellulose-decomposing bacteria with flexuous rods, which showed a creeping motility on solid surfaces. This definition was later expanded to include bacteria not capable of attacking cellulose (Stanier, 1940). More recently, with the advent of DNA base ratio determinations, the importance of gliding motility as an essential characteristic of *Cytophaga* has been questioned. The non-fruiting myxobacteria were found to generally have a low GC ratio in the 30s (Mandel and Lewin, 1969), while the fruiting myxobacteria had a higher GC ratio of about 70% (Mitchell, Hendrie and Shewan, 1969). However, determination of the GC ratios of a number of *Flavobacterium* species indicated that, while the base ratio of 'typical' flavobacteria was in the 60s, some non-swarming *Flavobacterium* spp., including the type species of the genus- *F. aquatile*, had low base ratios in the same range as those of *Cytophaga* species. In addition, certain flavobacteria capable of swarming had GC ratios of about 60% and a bacterium, which was seemingly a typical *Cytophaga* isolate, was found to have a GC ratio of 73.2% (Mitchell, Hendrie and Shewan, 1969).

Thus, either cytophagas were taxonomically heterogeneous, with a wide range of GC ratios, or swarming was not a property

restricted to cytophagas (Weeks, 1969). Mitchell, Hendrie and Shewan (1969) chose to place the most emphasis on GC ratios and proposed a new definition of the genus Cytophaga which included non-motile, yellow-pigmented bacteria, which although not showing gliding motility or able to degrade many polysaccharides, had a DNA base ratio in the range 29-45% GC. Lewin (1969), on the other hand, proposed a definition of Cytophaga which was nearer to the original concept of Cytophaga of Winogradsky (1929). Lewin restricted Cytophaga to those bacteria capable of gliding and usually able to digest many insoluble polysaccharides. Similar organisms morphologically but unable to attack a wide range of polysaccharides, were allocated to Flexibacter or Microscilla. However, there is no place in any of these three genera for non-motile flavobacteria with low GC ratios if they have short rods. The minimum length of any of the Cytophaga or Flexibacter species described is 5 μm and of Microscilla species, 20 μm.

The lack of a demonstrable cell wall has been cited as a distinguishing feature of Cytophaga (Breed, Murray and Smith, 1957). Hayes (1963) stained a number of Flavobacterium species and two presumptive Cytophaga species with the cell wall stain of Hale (1953). He found that, while distinct cell walls were seen in some of the flavobacteria, they were not visible in the cytophagas. However, a chemical analysis of the cell walls showed that there were certain similarities between the cell walls of the two groups of bacteria. Dworkin (1966) concluded that chemical and electron microscope studies of Myxococcus xanthus showed

that the cell wall of this fruiting myxobacterium was grossly similar to those of other Gram-negative bacteria.

However, some lower myxobacteria can be distinguished from eubacteria under the electron microscope. The surfaces of cells of Cytophaga johnsonii and Flavobacterium aquatile (now regarded as a Cytophaga species) appeared characteristically uneven when negatively-stained. Similar irregularities of the surfaces of other cytophagas have been reported (Colwell, Citarella and Chen, 1966; Bell, Hoskins and Hodgkiss, 1971). This appearance may serve to distinguish cytophagas from certain flavobacteria (Hodgkiss and Shewan, 1968).

Cytophagas have been reported in a number of aquatic habitats. Lewin and Lounsbery (1969) isolated gliding bacteria from both marine and freshwater habitats, which were mainly solid substrates. They identified these isolates according to the genera defined by Lewin (1969). All their Cytophaga spp. were of marine origin. Collins (1970) examined the bacterial flora of an oligotrophic lake and a eutrophic lake. The predominance of flavobacteria and cytophagas in waters that had been enriched from the surrounding land and from sewage was noted. It was suggested that the presence of myxobacteria might be an indicator of increasing enrichment of a lake. The presence of a yellow pigment and a gliding or flexing motility were two characters used to identify the cytophagas.

Ruschke and Rath (1965) studied a number of isolates of Sporocytophaga cauliformis isolated from lake water. They concluded that, as these myxobacteria could utilize a number of organic compounds in the laboratory, they would

be very active in the decomposition of organic matter *in situ*. The identification of this species is based mainly on the presence of microcysts. There is some doubt about the validity of this species as it has been suggested that the 'microcysts' are really spheroplasts which are frequently encountered in myxobacterial cultures (Dworkin, 1966; Hendrie, Mitchell and Shewan, 1968). However, these bacteria are myxobacteria of some kind and they would appear to be associated with higher levels of organic matter in lakes. Gräf and Stürzenhofecker (1965) distinguished two types of *S. cauliformis* on the basis of the strength of catalase activity and classified lakes according to the ratio of one type to the other. Schegg and Ruschke (1970) found that numbers of *S. cauliformis* in a mildly eutrophic lake were much higher than those in two other lakes which were subject to considerable faecal pollution. They did not distinguish between the two types of *S. cauliformis*.

Apart from making up a part of the water microflora, *Cytophaga* has also been reported to be predominant in the microflora of salmonid eggs (Bell, Hoskins and Hodgkiss, 1971; Trust, 1972). Bell, Hoskins and Hodgkiss noted that *Cytophaga* was most common on the surface of live eggs and made up only a small part of the microflora of dead eggs, simulated eggs, consisting of polyethylene spheres, and the stream water. Their isolates all had the typical surface appearance, reported by Follett and Webley (1965), and produced spreading colonies on agar. Myxobacteria were also predominant in the microflora of gills of a number of freshwater fish (Collins, 1970).

It appears that cytophagas may have special ecological niches in aquatic environments. In this chapter the identification of a group of bacteria which were mostly characterized by a spreading colony on agar and a yellow-orange pigment, is considered. Their presence in the samples is discussed in relation to the hypothesis of Collins (1970) that myxobacteria tend to be associated with enriched waters.

10.1 METHODS

Identification of isolates

a) as *Cytophaga*

More than 160 cytophagas were isolated during this study, of which 130 were characterized to varying extents. Nine of the isolates were tested intensively and are referred to in this chapter as 'the nine isolates'. The origin of these isolates is shown in Table 10.1.

Apart from the basic tests, described on pp. 34-36, which were used to characterize bacteria initially, a number of additional tests were carried out on some of the 130 isolates. Most of the media and methods used are described on p.47.

TABLE 10.1 Origin of nine isolates selected for further study

Date sampled	Sample	Isolate number
15/ 4/69	water over *Elodea*	1ST 191
25/11/69	water over harbour spring	5H 2
25/11/69	water over harbour spring	5H 4
20/ 8/70	water over *Elodea*	12W 1
20/ 8/70	water over *Elodea*	12W 2
9/ 9/70	open water	13D3 1
9/ 9/70	open water	13D3 2
9/ 9/70	net-concentrated plankton	13P 1
9/ 9/70	net-concentrated plankton	13P 2

Presence of phosphatase

Sensitivity to polymyxin B

Cellulose degradation: Some isolates were tested in the cellulose-mineral salts medium described on p. 36, but the nine isolates were tested further:-

(i) degradation of cellulose acetate;

(ii) After being maintained as stock cultures for up to two and a half years they were retested in the mineral salts medium with filter paper as the carbon source (p. 36). Bottles were incubated for 5 weeks;

(iii) At the same time, their ability to degrade filter paper in 'fermentation broth' was tested.

Morphology:

(i) spreading colonies - when cultures were first isolated on to GYCA the formation of spreading colonies was recorded. The nine isolates were also subcultured on to the low nutrient medium (yeast extract agar) described by Hendrie, Mitchell and Shewan (1968) and the ability of isolates to spread noted. Gliding motility of isolate 5H 2 on yeast extract agar was observed by placing a coverslip on the edge of a 24 h culture and examining with phase-contrast illumination;

(ii) dimensions of cells - isolate 13D3 2 was measured after 24 h in liquid AO medium, and 5 days in AO semi-solid agar (0.4% agar). Fifty cells were measured from both cultures;

(iii) presence of microcysts;

(iv) presence of a demonstrable cell wall - the nine isolates and cultures of Pseudomonas fluorescens and Escherichia coli from the Botany Department Culture Collection were stained;

(v) appearance under EM - cells of the nine isolates, cultured on nutrient agar slants for 24 h or 48 h at 20°C, were negatively stained as described on p. 34 and examined under the EM.

Identification of isolates

b) to species within Cytophaga

The following further tests, described on pp. 48-51, and mostly similar to those described by Lewin and Lounsbery (1969), were carried out on only the nine isolates:

Survival at +3°C;

Survival at -196°C;

Lauryl sulphate tolerance;

Tyrosine degradation;

Nitrate reduction;

Methyl cellulose degradation;

Starch hydrolysis;

Agar liquefaction;

Alginate liquefaction;

Gelatin liquefaction;

Catalase production;

Oxidase production;

Reaction in litmus milk medium;

Hydrogen sulphide production;

Salinity tolerance;

Penicillin tolerance;

Temperatures limits for growth;

Effect of temperature on growth rate;

Suitability of tryptone, casamino acids, sodium glutamate and KNO_3 as a nitrogen source;

Suitability of glucose, galactose, sucrose, glycerol, sodium acetate and sodium lactate as a carbon source;

Pigmentation.

The following tests described by Lewin and Lounsbery (1969) were not used in this study: anaerobic growth, chitin utilization, indole formation, sensitivity to 'Vibriostat' compound, production of ammonia - Lewin and Lounsbery did not use these tests in the evaluation of relationships between isolates because either they were difficult to interpret or they gave similar results with all strains - specific amino acid, vitamin or nucleic acid factor requirements - all the Cytophaga and Microscilla and half of the Flexibacter strains tested by Lewin and Lounsbery had no special requirements - determination of GC content of DNA and examination for the presence of rhapidosomes.

Evaluation of data: The data from the above tests were resolved into 58 features (Appendix 10), many of which were similar to those used by Colwell (1969) in her analysis of the data of Lewin and Lounsbery (1969). Similarity coefficients between the nine isolates and also Cytophaga lytica LIM-21 of Lewin and Lounsbery (1969), when tests were comparable, were calculated using the formula $S\% = \frac{ns}{ns + nd} \times 100$ in which negative matches are neglected (Sokal and Sneath, 1963). Isolates were grouped by the single linkage clustering technique.

Field samples

The presence of cytophagas on the pour plates of the field samples was recorded. When bacteria were not routinely isolated, a selection of bacteria with spreading, yellow-

orange colonies were isolated and their Gram reaction and morphology were noted.

The association of cytophagas with certain organisms was investigated:-

a) Plankton

The methods used to examine the microflora of algae and individual members of the zooplankton were described on p. 214.

b) Fish

The presence of Cytophaga on various parts of fish from Lake Grasmere was examined. Three trout - 1.6 kg male, 1.3 kg female brown (Salmo trutta) and 0.5 kg rainbow (Salmo gairdnerii) were caught in mid February, 1971, and placed in new plastic bags after capture. Samples from the following areas viz. slime from the side and tail, scales, gill filaments and anus, were streaked on to nutrient agar within 1 h of capture. An upland bully (Philypnodon breviceps) from an area 10-20 cm deep near the harbour was examined in early February, 1971. The side of the animal was swabbed and streaked on to agar and, after 1 h, a sample of the water the fish had been placed in was also streaked on to agar.

Enrichment for cellulose-degrading bacteria

Enrichment cultures from 58 samples collected between April, 1970, and February, 1971, were set up to select for cellulose-decomposing bacteria as described on p. 52.

10.2 RESULTS

Identification of isolates

a) as Cytophaga

All isolates produced a yellow-orange colony and had long, slender non-motile cells. The majority of the isolates tested were oxidase-positive, phosphatase-positive and had an oxidative utilization of glucose, although in most cases this was not apparent until after 7 days incubation (Table 10.2). The results of the tests for catalase production and polymyxin B resistance were more variable. None of the isolates tested could degrade filter paper or grow in the cellulose-mineral salts medium. Growth occurred in the AO fermentation broth but the filter paper was not attacked. There were no signs of any clearing of the cellulose acetate medium.

Not all isolates produced a spreading colony. In the earlier experiments a large number of cytophagas were isolated and 80 were recorded as not having a spreading colony. However, when further tests were carried out on some of these isolates, they did produce spreading colonies. When the nine isolates, which had previously shown spreading growth on GYCA and nutrient agar, were subcultured to the low nutrient medium only five spread across the agar. The edge of the colony of one isolate which had spread was examined with phase-contrast illumination and photographed at 5 minute intervals. Cells were seen to be moving away from the main colony when the photographs were examined but distinct gliding motility was not observed through the microscope.

TABLE 10.2 Biochemical characteristics of cytophagas

Test and result	No. tested	No. giving result
Oxidase-positive	130	128
Phosphatase-positive	20	19
Oxidative utilization of glucose	130	121
Catalase-positive	100	49
Polymyxin B resistant	20	12
Cellulose degraded	18	0

The length of the cells of isolate 13D3 2 varied from 2 to 5.5 μm with a mean of 3.4 μm after 24 h in liquid medium. The mean width was 0.4 μm. After incubation for 5 days in semi-solid agar the cells were shorter. The mean length was 1.8 μm and the width was 0.5 μm. No spherical bodies were seen in the cultures of the nine isolates after incubation for 5 days in semi-solid agar.

When the cell walls of a number of isolates were stained as described by Hale (1953), Pseudomonas fluorescens and Escherichia coli both stained green, but no clear region of cytoplasm could be distinguished. However, the cells of cytophagas stained were all a grey-blue colour. Under the EM some of the cells of all the nine cytophagas examined had wrinkled surfaces, with occasionally a few strands of material around the edges (Fig. 10.1). Cells which had a smooth surface typically had many more strands of material

FIG. 10.1 Electron micrograph of negatively-stained cells of Cytophaga no. 13P 1, showing 'wrinkled' appearance of some cells and strands around edges; x 32 000.

around the edges of the cell than there were around the 'wrinkled' cells. The appearance of cells from slants incubated for 24 h and 48 h was similar.

Identification of isolates

b) to species within Cytophaga

The results of tests used to calculate Similarity Coefficients between isolates are shown in Appendix 10. All the nine isolates from Lake Grasmere were grouped together at $\geq$85% S by the single linkage clustering technique (Fig. 10.2). However, Cytophaga lytica LIM-21 of Lewin and Lounsbery (1969), which, of all the flexibacteria of Lewin and Lounsbery, appeared to have the most characters in common with the Lake Grasmere isolates, did not join up until the 61% level.

Some tests gave inconclusive results. Liquefaction of alginate and methyl cellulose was difficult to assess as the media were not solid initially. None of the isolates could liquefy agar but all attacked starch and gelatin (Appendix 10). Promotion of growth by the carbon sources was in some cases only slight.

Tests for the highest temperature and the highest concentration of penicillin permitting growth both gave results outside the ranges considered in the analysis of Colwell (1969). The isolates from Lake Grasmere were resistant to much higher concentrations of penicillin and had lower temperature requirements (Appendix 10). Growth of the five isolates tested over a range of temperatures was most vigorous at 20°C. The next most favourable tempera-

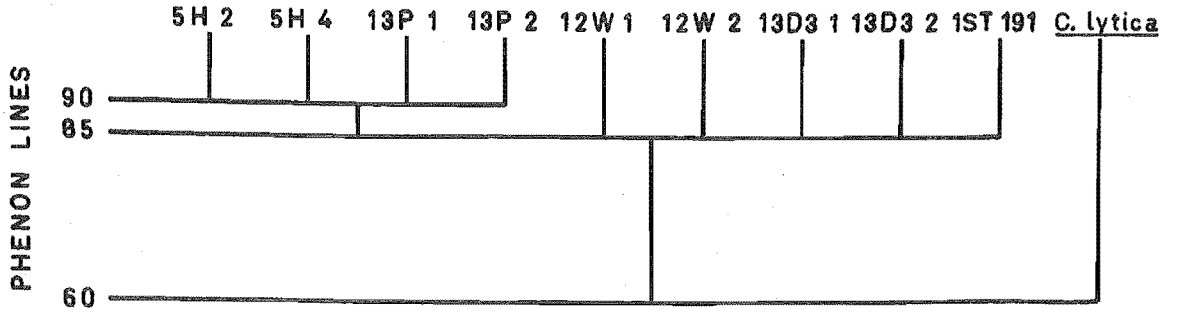


FIG. 10.2 Dendrogram of percent S values of Cytophaga isolates

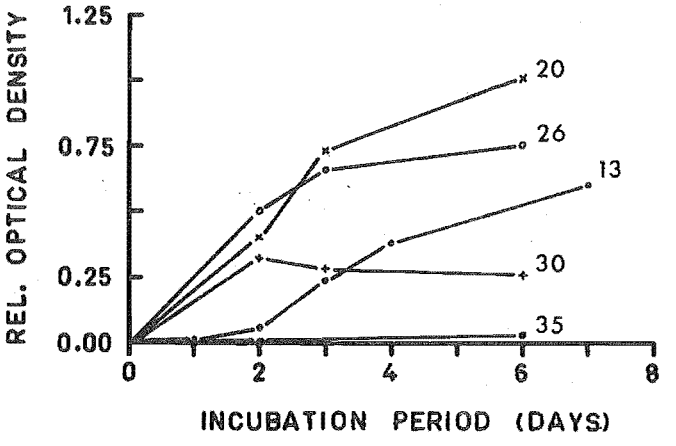


FIG. 10.3 Growth rate of Cytophaga no. 13P 1 at different temperatures (°C)

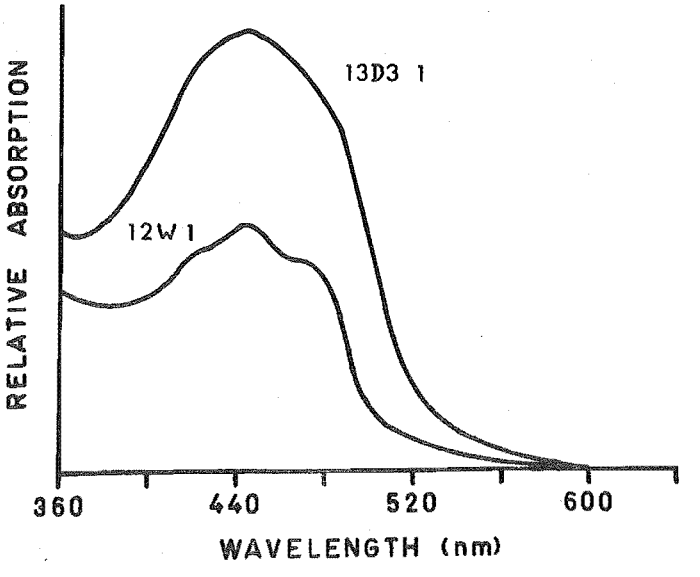


FIG. 10.4 Absorption spectra of pigment extracts in petrol ether.

ture was 26°C for three of the isolates and 13°C for the other two. The growth rate at the different temperatures of one isolate in the former group is shown in Fig. 10.3.

Two kinds of absorption spectra of the pigments in petrol ether were distinguished (Fig. 10.4). Isolates 12W 1 and 12W 2 had absorption spectra with a peak at 443-445 nm and two marked points of inflexion at 470-472 nm and 420-421 nm. The other 7 isolates had a single peak at 443-445 nm. There was little shift in wavelength of this latter peak when spectra were run in ethanol but the peaks of 12W 1 and 12W 2 both shifted about 5 nm so that the main peak was at 449 nm.

Field samples

The abundance of Cytophaga is shown in Table 10.3 and is represented by an arbitrary scale because of the difficulty of comparing absolute numbers of bacteria in different habitats such as water, mud and plankton.

Cytophagas were only found once on Elodea and then in very small numbers, and they were rarely found in the mud samples. Inlets A and C and stream E were only sampled on a few occasions and no cytophagas were found in the samples from A or C. However, a small number of cytophagas were isolated from a sample from stream E. In the water samples from over the harbour spring, over the weed and open water, cytophagas were found periodically but only occasionally did they occur in large numbers. Cytophaga made up the largest proportion of the bacteria characterized from any sample in

TABLE 10.3 Abundance of Cytophaga in field samples

Date sampled	Abundance in samples									
	Over Elodea	H	Open water	P	P_{pell}	P_{sn}	Elodea	Mud	A	C	E
15/ 4/69	++++		++					++			
17/ 6/69	++		++++					+			
28/10/69	-		-				-				
25/11/69		+++	+						-	-	+
17/ 3/70	-	-	-				-				
20/ 4/70		-	+								
19/ 5/70	+	+	+				-	-			
21/ 6/70	-	-	-				-	-			
7/ 7/70							-	-			
27/ 7/70	-	-	-				-	-			
20/ 8/70	++	+	-				-	-			
9/ 9/70	+	+	++	+			-	-			
9/10/70	+	++	+	+			-	+			
5/11/70	++	+	-	-			-	-			
9/11/70			-	+							
16/11/70			++	-							
23/11/70			+	-							
7/12/70	-	++	+	-	+	-	+	-			
5/ 1/71	-	+	+		+	+	-	-			
1/ 2/71	-	+	+		-	-	-	-			
24/ 2/71	+	+	-		-	-					-
8/ 3/71			-		-	-		-			-
22/ 3/71			-					-			-
7/ 4/71			-					-			
19/ 4/71			-								
3/ 5/71			-								
9/ 6/71			+		+	+					
13/ 7/71	-		-				-	-			

P - net-concentrated plankton; P_{pell} - centrifuged plankton;

P_{sn} - supernatant from centrifuged plankton; H - water over harbour spring.

- to ++++ arbitrary scale of abundance.

November, 1969, when 34% of the bacteria in a sample from over the harbour spring were Cytophaga. However, the largest number per ml was found in a sample of water over Elodea in April, 1969, when 21% of the bacteria characterized were Cytophaga which was equivalent to 230 per ml. Cytophagas were often found in several habitats at one time. They were not predominant in the plankton samples.

The abundance of Cytophaga in the samples appeared to be related to the year. Between October, 1969, and July, 1970, cytophagas were found in only a few of the samples. They were more common between August, 1970, and July, 1971, and were found in all the samples analyzed in April and June, 1969 (Table 10.3).

No cytophagas were found among the colonies appearing when individual plankton organisms were plated out (Table 7.7). Neither were there any cytophagas in the samples from different parts of the fish. Fluorescent pseudomonads were common, especially in the samples from the anus. Pink yeasts were conspicuous in the surface mucous samples from all three trout. Yeasts were rarely isolated from the lake water.

Enrichment

In only 10 of the 58 enrichment cultures was the filter paper attacked. In one case a Cytophaga was isolated from an enrichment culture but this species could not degrade cellulose when retested in the cellulose-mineral salts medium.

10.3 DISCUSSION

Identification of isolates

a) as *Cytophaga*

The characteristics of the bacteria designated *Cytophaga* from Lake Grasmere corresponded with the definition of *Cytophaga* proposed by Mitchell, Hendrie and Shewan (1969). Most were oxidase-positive and phosphatase-positive. None of those tested could attack filter paper, but the nine isolates tested in more detail could hydrolyze starch and liquefy gelatin (Table 10.2 and Appendix 10). Resistance to polymyxin B was, however, found in only 12 of the 20 isolates tested. It is possible that some isolates were sensitive because discs of 300 units were used instead of 100 units as used by Hendrie, Mitchell and Shewan (1968).

All the isolates designated *Cytophaga* were morphologically similar. They had long, slender cells. The dimensions of the isolate measured were within those recorded by Starr and Ordal (1953), Lewin and Lounsbery (1969) and Bell, Hoskins and Hodgkiss (1971). The difficulty encountered in this study in staining cell walls of cytophagas has been reported previously (Hayes, 1963). The irregular, 'wrinkled' appearance of the surface of cells visible with an EM (Fig. 10.1) may also be typical of cytophagas. Follett and Webley (1965) considered that the irregular surface topography was caused by slime contained within a flexible outer membrane. The presence of a slime layer is a characteristic of many cytophagas (Mitchell, Hendrie and Shewan, 1969). Strands appearing around some cells were considered

by Follett and Webley (1965) to be slime that had been extruded through pores in the membrane. These authors also suggested that negative-staining increased the extrusion of material because of the pressure of surface tension forces as the cells were dried on to the grid. Many strands can be seen around some of the negatively-stained cells in Fig. 10.1. Although insufficient observations have been made on flavobacteria to determine whether an irregular surface is widespread among these bacteria, it has been shown to be characteristic of a number of bacteria designated Cytophaga (Follett and Webley, 1965; Colwell, Citarella and Chen, 1966; Hodgkiss and Shewan, 1968; Bell, Hoskins and Hodgkiss, 1971).

Not all the cytophagas isolated were recorded as producing spreading colonies. However, some produced spreading colonies erratically, seeming to 'lose' the ability to spread and then regaining it when subcultured on another occasion. Nevertheless, the bacteria designated Cytophaga in this study formed a distinctive group and were readily identified as Cytophaga on the basis of the definition of Mitchell, Hendrie and Shewan (1969). However, the isolates did not conform to the definition of Cytophaga suggested by Lewin (1969), as they could not digest cellulose in the form of filter paper, and liquefaction of methyl cellulose was only slight. Lewin allocated freshwater isolates, which were unable to attack cellulose to the genus Flexibacter. In the next section, the results of further tests described by Lewin and Lounsbery (1969) on the nine isolates from Lake Grasmere are given, and the relationship of these isolates to Cytophaga and Flexibacter spp. of Lewin and Lounsbery is assessed.

Identification of isolates

b) to species within Cytophaga

Mitchell, Hendrie and Shewan (1969) considered that a satisfactory key to species of Cytophaga could not be given. However, Lewin (1969) has designed a key to species of flexibacteria based on the data of Lewin and Lounsbery (1969) which were analyzed by Fager (1969) using a recurrent group analysis. An Adansonian analysis of some of this data was carried out by Colwell (1969).

Forty-nine features similar to those recognized by Colwell (1969) were used in the present analysis. Another 9 features from results of tests described by Lewin and Lounsbery (1969), which were not used in the analysis of Colwell, were also included (Appendix 10). Using single linkage sorting, the isolates from Lake Grasmere showed a high degree of similarity, being grouped together at $\geq$ 85% S. However, Cytophaga lytica of Lewin and Lounsbery (1969) did not join up until the 61% level (Fig. 10.2). In the Adansonian analysis of the isolates of Lewin and Lounsbery, all isolates designated Cytophaga, Microscilla or Flexibacter were grouped together at $>$ 80% S. Colwell (1969) noted that the 67 features used in her analysis represented a minimum degree of information suitable for Adansonian methods and there was a similar limitation in the present study as only 58 features were analyzed. However, the low Similarity Coefficients between the Lake Grasmere isolates and Cytophaga lytica LIM-21 indicated that the freshwater isolates were distinct from the marine cytophagas studied by Lewin and Lounsbery (1969). Furthermore, the characteristics of

the Lake Grasmere isolates did not correspond to any of the groups recognized by Fager (1969), which were based on his recurrent group analysis of the data of Lewin and Lounsbery (1969). These groups included species of both Cytophaga and Flexibacter.

Some of the tests of Lewin and Lounsbery were difficult to interpret or gave results outside the range obtained by these workers. The substrate used by Lewin and Lounsbery (1969) to determine cellulase activity was carboxymethyl cellulose. In the present study methyl cellulose was used. Apart from problems of assessing the degree of liquefaction, interpretation of positive results was also difficult. Lewin and Lounsbery noted that none of their strains clearly degraded filter paper and similar results were obtained in this study. It is hard to determine the significance of possible attack on carboxymethyl cellulose or methyl cellulose when filter paper, which is more commonly used to test cellulase activity, is not digested.

Lewin and Lounsbery (1969) noted that the tests for suitable carbon sources for growth were not always reproducible and, although Colwell (1969) included these data in her analysis, Fager (1969) did not. The method used depended on growth being stimulated by the added carbon source over that on the control plates. Lewin and Lounsbery found that their isolates were very fastidious and included supplements such as tryptone in the basal medium. Sodium glycerophosphate was also present which would be a carbon source. The isolates from Lake Grasmere proved to be less fastidious and some grew quite vigorously on the control plates. However, this may have been due to the

presence of glycerol which was included at a low concentration in the basal medium. A positive result was therefore recorded only when growth on the test plates were distinctly greater than that on the control plates. It is possible that some positive results could have been caused by the autoclaving of the carbon sources, as recommended by Lewin and Lounsbery, which might have released some compounds suitable for growth. As the results were not very satisfactory, production of acid from the same carbon sources, which had been filter-sterilized, was also tested twice using a peptone medium with phenol red as the indicator. However, the results of these tests were not included in the analysis as they were not reproducible and often an alkaline reaction was obtained or the medium turned orange.

Lewin and Lounsbery (1969) found that their isolates often grew faster at 30°C or 35°C than at room temperature. In the present study, growth of the isolates tested at 35°C was very slight and, at 30°C, was slower than at 26°C or 20°C.

The absorption spectra of the pigments of the Lake Grasmere cytophagas were not identical to any of those described by Lewin and Lounsbery (1969). However, the wavelengths of the peaks of their Type 1V pigment were close to those of isolates 12W 1 and 12W 2 and in the Adansonian analysis of results, the pigments of these two cytophagas were considered to be identical to Type 1V. McMeekin, Patterson and Murray (1971) examined the absorption spectra of a range of yellow-pigmented bacteria and the three peaks

of their Type III pigment were close to those of 12W 1 shown in Fig. 10.4. A curve similar to that of 13D3 1 was not recorded. These authors discussed the taxonomic significance of the type of pigment. They noted that the majority of groupings based on biochemical characteristics were confirmed by the pigment analyses. However, the pigments of some strains were intermediate between two groups and some divisions based solely on absorption spectra were not compatible with other results such as DNA base ratios. They concluded that there were serious limitations to the use of this parameter at the generic level.

With the low level of similarity between the isolates from Lake Grasmere and Cytophaga lytica LIM-21 the key of Lewin (1969) to species of Cytophaga was not applicable. Neither were any of the Flexibacter spp. of Lewin and Lounsbery (1969) similar to the isolates from Lake Grasmere. The Lake Grasmere isolates formed a group which was so distinct biochemically from the flexibacteria of Lewin and Lounsbery, that a number of their tests could not be used without modification. As the isolates from Lake Grasmere were not identical to any of the Flexibacter spp. of Lewin and Lounsbery, it was preferable to consider them as species of Cytophaga as defined by Mitchell, Hendrie and Shewan (1969). However, a satisfactory key to species of these cytophagas has yet to be developed. The isolates, on which additional tests were carried out, might all be considered as strains of one species but too few isolates were characterized fully for key characters to be differentiated. In this

study isolates could only be identified to genus.

Field samples

Previous work has shown that Cytophaga is commonly part of the microflora of salmonid eggs (Bell, Hoskins and Hodgkiss, 1971; Trust, 1972). Collins (1970) found that myxobacteria were the predominant bacterial group in samples from gill filaments of healthy freshwater fish, including brown and rainbow trout, and concluded that this site was particularly suitable for slime-producing bacteria. An analysis of samples from trout and an upland bully from Lake Grasmere failed to demonstrate any cytophagas. Only a very small number of fish, and not all the species found in Lake Grasmere, were examined but the data do not suggest that fish are generally a source of cytophagas in the lake water. The possibility that cytophagas might be concentrated on fish eggs was not investigated. However, as there is no suitable inlet stream for trout to spawn in, some spawning probably occurs in the lake between April and October. Some cytophagas found in the lake water might have originated from eggs, but as cytophagas were found at intervals throughout the year they must either have been able to survive in lake water for some time or have come from other sources in the lake.

The microflora of Elodea and plankton was also examined. Samples of concentrated plankton did not contain any more cytophagas than the untreated lake water and in only one sample of Elodea did Cytophaga make up a part of the microflora. Maceration of a suspension of a Cytophaga isolate

showed that its absence in the Elodea samples was not due to the method used to plate out the bacteria (p.180).

Cytophagas were found in the mud samples in April and June, 1969, and in October, 1970. At these times they were also abundant in the lake water, suggesting that suitable nutrients for Cytophaga were present throughout the lake.

Cytophaga did not appear to be consistently associated with Elodea, plankton, mud or fish, but was found frequently in the water samples although often in low numbers. Cytophagas were not isolated from the cellulose enrichment cultures so it is unlikely that they were decomposing cellulose in situ. Collins (1970) suggested that myxobacteria might be indicators of increasing enrichment of lakes and the myxobacterium Sporocytophaga cauliformis is thought to be active in the decomposition of organic matter in lakes (Ruschke and Rath, 1965).

In general, Cytophaga made up only a small part of the bacterial population of the water during the period the lake was sampled. This would confirm Collins' hypothesis as the trophic state of Lake Grasmere is considered to be oligotrophic to mildly eutrophic. Bacteria similar to S. cauliformis were not found in Lake Grasmere. If the predominance of Cytophaga in samples taken over a period of time is an indicator of the trophic state of a lake, it is also possible that the seasonal distribution, and horizontal distribution within the lake, might provide measures of fluctuating nutrient concentrations. In the main body of the lake, Cytophaga was most common in April and June,

1969 (Table 10.3). Their abundance coincided with higher numbers of bacteria per ml compared with the same periods in the following two years. In Chapter 4 the relationship between rainfall during the previous months and the numbers of bacteria in the open water was discussed. It was suggested that a greater inflow of nutrients had caused the higher numbers of bacteria found in April and June, 1969, compared to 1970 and 1971 (Table 4.2; p. 131). By the same argument the abundance of Cytophaga in 1969 might have been related to a higher nutrient level. Cytophaga was also found in the open water or over the weed from August, 1970, to February, 1971. Its appearance in August and September coincided with the heavy rains, when nutrients would have been brought into the lake.

It thus appeared that the presence of Cytophaga might be related to the inflow of nutrients. The proportions of Cytophaga in some of the inlets were examined to determine whether Cytophaga was being brought into the lake with the inflowing water. No cytophagas were found in inlet C, which typically had a much larger bacterial population than the open water, or inlet A. The single sample from stream E included a small number of Cytophaga but Cytophaga did not make up a large part of the microflora of the samples from inlets A or C, or stream E.

Cytophagas were frequently found in the samples from over the harbour spring. They made up the highest proportion in November, 1969, when there was little inflow of water and particulate matter as rainfall in the 7 days before sampling was very low. This suggested that it was

the nature, rather than the quantity, of the nutrients flowing into the harbour which was selecting for Cytophaga. It is possible that Cytophaga was able to compete for any organic matter present because it could utilize certain minerals brought in through this inlet more successfully than other bacteria. The Cytophaga isolate tested was able to multiply in filter-sterilized lake water in 24 h, and survived in filter-sterilized lake water for the duration of the experiment (Table 6.3).

In March and April, 1970, Aeromonas/Vibrio and Enterobacteriaceae were common and it was suggested that this was due to the presence of birds on the lake and that a higher level of organic matter might be expected in the lake water (p.133). At this time Cytophaga was almost completely absent. This also suggested that the presence of organic matter was not the main factor controlling the predominance of Cytophaga. The relationship between the cytophagas, which may be responding to increased enrichment of a lake, and those likely to be present on fish eggs has still to be determined.

The data available suggested that the presence of Cytophaga might be an indicator of increased enrichment of a lake as hypothesized by Collins (1970). However, it appeared that the predominance of Cytophaga in Lake Grasmere might depend on the addition of certain unspecified minerals rather than on the concentration of organic matter.

10.4 SUMMARY

1) The identity of a number of isolates designated Cytophaga was considered. Many produced spreading colonies on agar. When nine isolates were negatively-stained and examined with an EM, they were found to have an irregular surface. None of the Cytophaga isolates tested could degrade filter paper, and no cellulose-decomposing cytophagas were isolated from enrichment cultures with filter paper as the carbon source.

2) Nine Cytophaga isolates were tested intensively. They formed a group which was distinct from the Cytophaga and Flexibacter spp. of Lewin and Lounsbery (1969).

3) Cytophaga was rare in the mud, on Elodea and in inlets C and A. It was not found on the four fish examined (trout and upland bully). Cytophagas were, however, found more frequently in the lake water and over the harbour spring, but in general they made up only a small part of the bacterial population. This did not controvert the hypothesis of Collins (1970), as the lake is considered to be oligotrophic to mildly eutrophic.

4) It was suggested, after an analysis of the abundance of cytophagas in all the samples collected, that the presence of certain kinds of nutrients, such as minerals and trace elements, rather than the quantity of nutrients, such as organic matter, was controlling the predominance of Cytophaga.

CHAPTER 11

THE ECOLOGY OF VIBRIO EXTORQUENS

A number of the samples from Lake Grasmere contained bacteria which formed small pink colonies on the pour plates. Apart from their pigmentation, these bacteria were distinct morphologically as they had, at one or both ends of a cell, a large granule which did not stain with the Gram stain.

These bacteria were identified as methanol-utilizing bacteria which have been reported a number of times (Kaneda and Roxburgh, 1959; Harrington and Kallio, 1960; Hayward, 1960; Peel and Quayle, 1961; Anthony and Zatman, 1964; Stocks and McClesky, 1964; Shul'govskaya, Andreeva and Rabotnova, 1971). Stocks and McCleskey (1964) compared strains of pink-pigmented, methanol-utilizing bacteria. They noted that the majority of the pink-pigmented bacteria could grow on peptone media without the addition of other carbon sources and could not oxidize methane. However, Dworkin and Foster (1956) and Leadbetter and Foster (1958) isolated morphologically similar bacteria, which were obligate utilizers of methane or methanol. Since then there have been varying opinions on whether bacteria which can oxidize methane and methanol can only do so obligately. Overbeck and Ohle (1964) considered that a range of heterotrophic bacteria could oxidize methane, but in their review Ribbons, Harrison and Wadzinski (1970) stated that all methane-oxidizers so far studied could only grow in the presence of methane or methanol although they might be able to

oxidize common metabolites, such as glucose, and incorporate them into cellular constituents during growth on methane. It seems likely therefore that pink-pigmented bacteria which can grow on complex organic media in the absence of methane and can utilize methanol as a sole carbon source will be unable to oxidize methane.

The main problem which arises from the question of whether these bacteria can oxidize methane is their nomenclature. From the above discussion it appears that the pink-pigmented, methanol-utilizing bacteria are distinct from the obligate methane-oxidizers and this should be clear in their nomenclature. The name Pseudomonas methanica has been applied to both pink-pigmented bacteria which cannot utilize methane (Harrington and Kallio, 1960) and those that can (Dworkin and Foster, 1956). Stocks and McCleskey (1964) discussed the taxonomy of the methanol-utilizing bacteria and suggested that they should be considered strains of Vibrio extorquens (Bassalik)(Bhat and Barker, 1948) and this nomenclature is followed in the present study.

In most of the studies of methanol-utilizing bacteria, isolates have been obtained from enrichment cultures of methane- or methanol-oxidizing bacteria. The inocula for these enrichment cultures have been diverse : soil (Kaneda and Roxburgh, 1959; Harrington and Kallio, 1960; Anthony and Zatman, 1964; Shul'govskaya, Andreeva and Rabotnova, 1971); rumen of a cow, coal-mine waters and oil field soil (Stocks and McCleskey, 1964); and a eutrophic lake (Naguib and Overbeck, 1970). Pink-pigmented, obligate methane-oxidizers have been isolated from freshwater pond mud and Elodea

(Dworkin and Foster, 1956; Leadbetter and Foster, 1958). Naguib and Overbeck (1970) did not name their isolates, but their 'V form' was pink-pigmented and could utilize methanol but not methane, and was probably a Vibrio extorquens strain. Hayward (1960) discussed the identity of pink-pigmented bacteria, which could utilize methanol and had been isolated from tropical plant material. He did not record whether they were initially isolated from enrichment cultures.

Apart from the study by Naguib and Overbeck (1970), pink-pigmented, methanol-oxidizing bacteria have not been reported in lakes. This may be because they are normally present in very low numbers in lake water so they can only be isolated by enrichment, or alternatively they may not have been recognized as methanol-utilizers. Snow and Fred (1926) and Graham and Young (1934) noted that red and pink colonies were the next most commonly found chromogens on plates of lake water samples after yellow- and orange-pigmented bacteria. Potter and Baker (1961) found pink-pigmented bacteria in all samples from Flathead Lake and occasionally in samples from the more eutrophic Rogers Lake.

In this chapter some features of the isolates of Vibrio extorquens from Lake Grasmere are noted. The abundance of Vibrio extorquens in the samples from Lake Grasmere and its inlets collected over two and a half years is considered and some reasons for this abundance are discussed.

11.1 METHODS

Apart from the routine tests described on pp. 34-36, the following tests described on p. 51 were carried out on some of the pink-pigmented bacteria isolated during this study:-

Staining for spores,

Staining for fat droplets,

Utilization of methanol as a sole carbon source : Eighteen Vibrio extorquens isolates and five other bacteria - a fluorescent pseudomonad, flavobacterium, Cytophaga, coryneform and a pink-pigmented, Gram-positive coccus - were streaked on to the test plates.

Four other Vibrio extorquens isolates were tested in liquid medium.

Effect of temperature on growth rate

One isolate was subcultured to bottles of nutrient broth which were incubated at 20±1, 25±2, 29±2, 35±2°C. Turbidity was noted at intervals for 16 days.

Field samples

The presence and abundance of pink-pigmented bacteria on the pour plate of the field samples was recorded. When bacteria were not routinely isolated, a selection of pink-pigmented colonies was isolated and the Gram reaction and motility determined. The ability of isolates to utilize methanol as a sole carbon source was tested in some cases.

The presence of Vibrio extorquens in the microflora of fish and its association with planktonic organisms was examined in the experiments described on p. 301 and p. 214 respectively.

Enrichment for methanol-utilizing bacteria

Enrichment cultures were set up as described on p. 52 from all samples collected on 11 sampling dates between May, 1970, and February, 1971.

11.2 RESULTS

More than 110 pink-pigmented bacteria which were identified as Vibrio extorquens were isolated. Other red-pigmented bacteria were found in the samples but these could usually be distinguished from Vibrio extorquens by their darker red or orange-red pigment. These bacteria were either coryneforms or Gram-positive cocci. Vibrio extorquens always grew slowly on GYCA when isolated from the pour plates and often colonies were scarcely visible after incubation for 4 days. When incubation was extended to 1 or 2 weeks colonies were well developed.

All isolates were Gram-negative and had conspicuous granules which did not stain with the Gram stain (Fig. 11.1). The motility of cultures varied. Some isolates were non-motile when 24 h nutrient broth cultures were examined but usually a few cells were motile. Negative-staining and examination under the EM showed that the bacteria had single

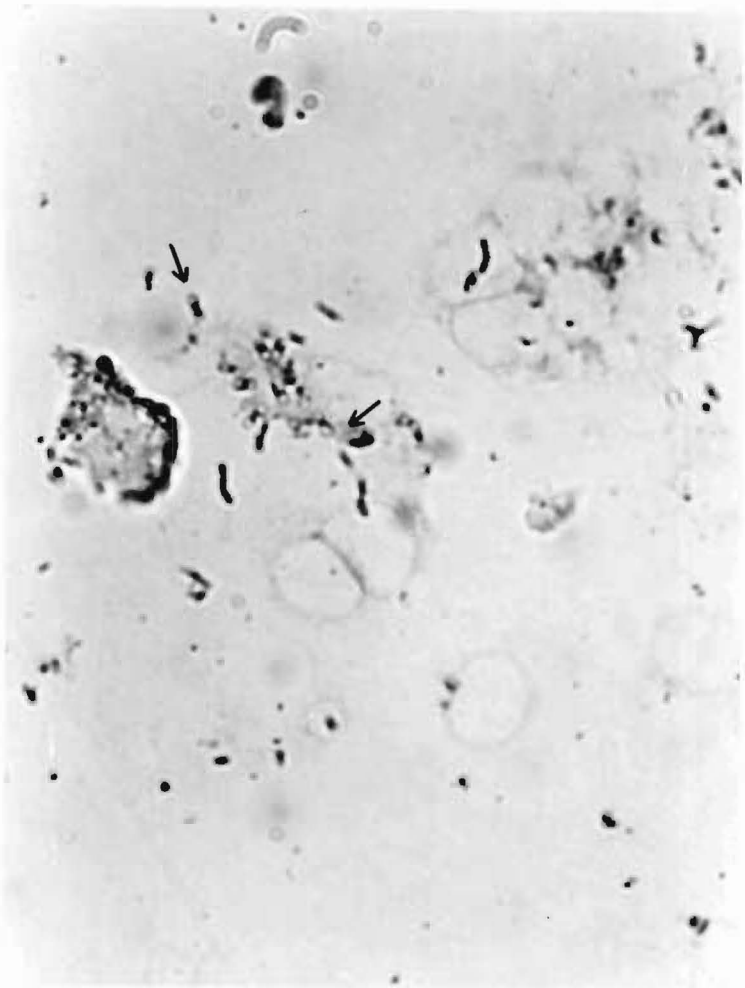


FIG. 11.1 Gram-stained cells of Vibrio extorquens, showing unstained granules at ends of cells (arrowed); x 1 650.

polar flagella and that the granules which were distinct under the light microscope were not visible under the EM. The catalase test was generally positive, while oxidase varied, with many of the isolates having a delayed positive reaction. The ability to produce ammonia fermentatively from arginine was tested in some of the cultures and all were negative. All isolates tested for cellulose utilization were negative. Many isolates could not produce acid from glucose in the medium of Hugh and Leifson (1953), although they did show growth. Some utilized glucose oxidatively after 7 to 14 days incubation.

Stains: The granules did not stain with either of the spore stains used. However, the granules did take up Sudan black B, indicating that they consisted of lipids.

Utilization of methanol: All isolates tested grew on GYCA and did not grow on the control medium without methanol. All *Vibrio extorquens* isolates grew on the methanol medium. Some showed more vigorous growth than others but only one grew more vigorously on the methanol medium than on GYCA. None of the control bacteria grew on the methanol medium. The four isolates tested in liquid methanol medium of Stocks and McCleskey (1964) grew quite vigorously in the medium with added methanol and showed no turbidity in the control bottles with no methanol.

Effect of temperature on growth rate

The isolate showed little growth for the first three days of incubation at any of the temperatures, but then grew well at 20°C and 25°C (Table 11.1). The growth occurring

during the latter part of the experiment at 29°C was probably due to a slight drop in the temperature of the incubator.

TABLE 11.1 Effect of temperature on the growth rate of Vibrio extorquens

Temperature (°C)	Incubation period (days)				
	2	4	5	8	16
20 ± 1	(+)	++	+++	++++	++++
25 ± 2	(+)	+	+++	++++	++++
29 ± 2	-	-	-	(+)	++
35 ± 2	-	-	-	(+)	(+)

(+) slight turbidity

- to ++++ arbitrary scale of turbidity

Field samples

The abundance of Vibrio extorquens on the pour plates is shown in Table 11.2 and is represented by an arbitrary scale because of the difficulty of comparing absolute numbers of bacteria in different habitats such as water, mud and plankton. In the water samples Vibrio extorquens made up 26% of the bacteria characterized from the open water in April, 1969. The largest number per ml of lake water was 190 per ml in the water over weed sampled in June, 1969.

Vibrio extorquens was not found in the mud or on Elodea.

TABLE 11.2 Abundance of Vibrio extorquens in field samples

Date sampled	Abundance in samples										
	Open water	P	P_{pell}	P_{sn}	Over Elodea	H	Elodea	Mud	A	C	E
15/ 4/69	+++				+			-			
17/ 6/69	+				+++			-			
28/10/69	-				-		-				
25/11/69	-					-			-	+	-
17/ 3/70	-				-	-	-				
20/ 4/70	-					-					
19/ 5/70	-				-	-	-	-			
21/ 6/70	-				-	-	-	-			
7/ 7/70							-	-			
27/ 7/70	-				-	-	-	-			
20/ 8/70	-				-	-	-	-			
9/ 9/70	-	+			-	-	-	-			
9/10/70	+	++			+	-	-	-			
5/11/70	+	++			-	-	-	-			
9/11/70	++	++									
16/11/70	-	++									
23/11/70	++	++									
7/12/70	-	+	++++	++	-	-	-	-			
5/ 1/71	-		-	-	-	-	-	-			
1/ 2/71	-		-	-	-	-	-	-			
24/ 2/71	-		+++	++	-	-				-	
8/ 3/71	-		-	-				-		-	
22/ 3/71	-									-	
7/ 4/71	+							-			
19/ 4/71	+++										
3/ 5/71	+++		+++	+							
9/ 6/71	+		-	-							
13/ 7/71	+		-	+	-		-	-			
5/12/71	-										

P - net-concentrated plankton

P_{pell} -centrifuged plankton

P_{sn} -supernatant from centrifuged plankton

H -over harbour spring

- to ++++ arbitrary scale of abundance

It appeared spasmodically in the lake water and plankton samples. During the period of study there were three times when Vibrio extorquens was conspicuous - April and June, 1969; September to December, 1970, and April to July, 1971. At these times it was not found in all the water samples taken on the same date and did not occur in any of the samples from over the harbour spring. However, harbour samples collected at the same times by Dr V.M. Stout in which numbers of bacteria were also estimated by the pour plate method, occasionally showed Vibrio extorquens. It was notable that Vibrio extorquens was often found in the plankton samples. All the net-concentrated plankton samples examined included some Vibrio extorquens and when it was found in the centrifuged plankton samples there was always a smaller proportion in the supernatant fraction. However, not all centrifuged plankton samples included Vibrio extorquens and experiments to determine whether the planktonic organisms had a specific microflora indicated that it was doubtful if any of the bacteria normally cultured were directly associated with the plankton (p.230 and Table 7.7). Several Vibrio extorquens grew from two algal samples studied in November and December, 1970 (Table 7.7). At this time Vibrio extorquens was common on the plates used to enumerate the bacteria in the plankton samples. However, it was not the dominant bacterium in the algal samples and was not associated with any of the animals examined (Table 7.7). Vibrio extorquens did not occur in any of the samples from the trout and the bully (p.309).

Enrichment cultures

Although individual isolates of Vibrio extorquens could grow in the methanol medium of Stocks and McCleskey (1964), Vibrio extorquens was not isolated from any of the enrichment cultures set up, even at the times when the pour plates showed colonies of this bacterium. In cases where bottles became turbid, no pink colonies grew when samples were streaked on to nutrient agar. Enrichments from mud and Elodea which were incubated at 20°C and 30°C produced no pink-pigmented bacteria. Twelve bacteria were isolated from the first set of enrichment cultures from five different habitats. None was pink-pigmented, but one, which was a fluorescent pseudomonad, was able to utilize methanol as a sole carbon source.

11.3 DISCUSSION

Identity of isolates

The pink-pigmented bacteria isolated from Lake Grasmere were characterized by the presence of sudanophilic granules and the ability to utilize methanol as a sole carbon source. They could also grow on more complex organic media as could the isolates tested by Stocks and McCleskey (1964). These authors found all their isolates were catalase-positive and oxidase-negative. In this study the majority of the isolates were catalase-positive, but some were oxidase-positive or had a slow positive reaction.

All the pour plates and enrichment cultures were routinely incubated at 20°C and, as might be expected, the isolate tested grew well at 20°C and 25°C. However, little growth occurred at 29°C. Almost all enrichments for methanol-oxidizing bacteria reported in the literature have been incubated at 28°C or 30°C. Where isolates were tested at several temperatures, optimum growth was recorded at 30°C, with none at 37°C and slow growth at 23°C to 25°C (Kaneda and Roxburgh, 1959; Peel and Quayle, 1961). Harrington and Kallio (1960) incubated their cultures at 25°C but did not mention if isolates were tested at other temperatures. Thus, except possibly for the isolate of Harrington and Kallio (1960), the isolates previously recorded have different temperature requirements from the Lake Grasmere isolates.

The lack of success in isolating Vibrio extorquens from enrichment cultures was unexpected. It is possible that the nutrients from the inoculum were sufficient to allow the growth of bacteria, which could not utilize methanol, and these grew over the methanol-utilizers when samples were streaked on to a non-selective agar. Fresh methanol medium should have been inoculated with the initial enrichment culture. However, in some bottles there was little growth of any bacteria and it is possible that certain growth factors were required in the enrichment medium. While the four isolates tested grew in the medium of Stocks and McCleskey (1964), these isolates were subcultured from nutrient agar and sufficient growth factors might have been carried over to ensure growth. Any transfer of nutrients was not completely

responsible for the growth as none occurred in the control bottles. Growth in the methanol medium was not as vigorous as in nutrient broth. The methanol-utilizing bacteria of Peel and Quayle (1961) and Anthony and Zatman (1964) grew in mineral media after serial dilution with no added growth factors, but the isolate of Kaneda and Roxburgh (1959) required biotin.

Thus, the bacteria isolated from Lake Grasmere differed from those previously described in their temperature requirements, and possibly in a requirement for growth factors and their oxidase reaction. However, with their ability to utilize methanol as a sole carbon source and their morphological similarity to the strains of Vibrio extorquens, described by Stocks and McCleskey (1964), it seems likely that the Lake Grasmere isolates are strains of the same species.

Presence in field samples

The erratic appearance of Vibrio extorquens in some of the field samples raised several questions, the answers to which can only be suggested. The basic problem was whether these bacteria were utilizing simple carbon compounds in the lake. If they were, what was the source of these compounds? Stocks and McCleskey (1964) found that their isolates could grow equally well on methanol, glycerol or oxalate.

As the growth of Vibrio extorquens was slow on complex organic media, it is likely that these bacteria would be better able to compete for simple carbon compounds. The ability of the isolate, tested in filter-sterilized lake

water, to increase six-fold in 24 h (Table 6.3) indicated that Vibrio extorquens was an efficient utilizer of dissolved nutrients. This would suggest that it is particularly suited to growth in a dilute nutrient system such as a lake. However, Vibrio extorquens was only found irregularly in the lake water, suggesting it required a special set of conditions before it became conspicuous. These conditions might include the presence of simple carbon compounds.

If Vibrio extorquens is dependent on methanol to be able to compete successfully, one possible source could be from bacterial oxidation of methane formed anaerobically in the mud. As discussed in Chapter 9, aerobic decomposition in the mud does not appear to be taking place rapidly in Lake Grasmere. If nutrients, e.g. phosphate, became available when there was an increased rate of sedimentation of organic matter on to the mud, for example, after some dying off of Elodea in autumn, some anaerobic decomposition would most likely take place. For methane to be converted to methanol, oxygen is required and this might be the reason why no Vibrio extorquens was found in the mud samples.

Thus, one possibility is that the presence of Vibrio extorquens could be related to the level of anaerobic decomposition in the mud. It may be that a combination of lack of oxygen in the mud, but well-oxygenated lake water is required for this bacterium to become dominant.

Obligate, methanol- or methane-oxidizing bacteria, which were pink-pigmented, have been isolated from Elodea growing in a shallow pond (Dworkin and Foster, 1956). The

lack of Vibrio extorquens on Elodea in Lake Grasmere may have been due to the well-oxygenated state of the weed beds when there would be little anaerobic decomposition of the plant with the formation of methane.

Little information is available on the occurrence of this bacterium in other lakes, but on pour plates of water samples, collected by Dr V.M Stout from a very different lake (Lake Mapourika), there were large numbers of Vibrio extorquens. Lake Mapourika is distinctly oligotrophic and is deeper (80 m) than Lake Grasmere. At the time of sampling the lake was beginning to stratify but was still well oxygenated.

In one eutrophic lake in which methane-oxidizing bacteria were studied specifically, Naguib and Overbeck (1970) were unable to isolate any obligate methane-oxidizers although bacteria, including some like Vibrio extorquens, were isolated from enrichments under methane. These pink-pigmented bacteria could not oxidize methane but could utilize methanol. The lack of any methane-oxidizers in these enrichments was surprising. It may have been due to the techniques used, or else it could be that the methanol-oxidizing bacteria were not dependent in the lake on methanol formed from methane by bacterial oxidation.

An alternative source of carbon for Vibrio extorquens might be provided directly by the plankton. Although the Vibrio extorquens isolates from Lake Grasmere were not tested, glycerol and oxalate were utilized as readily as methanol by the Vibrio extorquens strains of Stocks and McCleskey (1964). Studies of the extracellular products of algae

have shown that both oxalic acid and glycerol may be excreted (Fogg, 1971). Another carbon source might be glycollic acid, which is well-documented as a product of photosynthesis (Fogg, 1971) and has a formula related to those of methanol, glycerol and oxalic acid. Wright (1970) found that glycollate uptake by planktonic bacteria was comparable with that of glucose and acetate uptake. However, Vibrio extorquens was not always found in the plankton samples and there was no apparent association with any particular alga or animal. This might be because excretion of extracellular products by algae is affected by the environmental conditions (Fogg, 1971). It is possible that Vibrio extorquens was only found in the plankton samples because it was either associated with some particulate matter or grew in clumps so that it tended to be concentrated with the plankton.

Thus, there are a number of ecological niches into which Vibrio extorquens might fit. The present study has only been able to show that this bacterium is occasionally a conspicuous part of the microflora of this lake and suggest some reasons for the irregular appearance of this bacterium. Whether Vibrio extorquens has a special role in lakes has yet to be determined.

11.4 SUMMARY

1) Pink-pigmented bacteria, identified as Vibrio extorquens, were occasionally common in the lake. They often made up a larger part of the microflora of the concen-

trated plankton than of the untreated lake water.

2) The possibility that this species might be utilizing methanol, or products from algae, in the lake, is discussed.

CHAPTER 12

THE ROLE OF BACTERIA IN THE MAINTENANCE OF NUTRIENT LEVELS IN LAKE GRASMERE

The data obtained during this study showed that the numbers of bacteria in the lake water were low and, generally, there was little response by these bacteria, or the benthic bacteria, to the addition of organic matter in the form of plankton or Elodea. This suggested that the nutrients bound in these organisms were not being rapidly mineralized by bacteria in either of these habitats in the lake. The availability of minerals, such as phosphorus, or trace elements may have been limiting bacterial activity. Two main questions arise from these observations:

(i) how are concentrations of minerals maintained at a high enough level to support the considerable standing crop of Elodea canadensis and large populations of algae in the lake; and

(ii) if sufficient quantities of these nutrients are available for the primary producers, why are bacteria apparently unable to utilize them, and rapidly decompose any organic matter present?

In this chapter the relative importance of bacteria in the mineralization of nutrients, and some possible mechanisms which might be limiting bacterial activity in Lake Grasmere, are discussed.

12.1 METHODS OF RECYCLING NUTRIENTS IN AN AQUATIC ENVIRONMENT

In Chapter 1 it was noted that although the recycling of such nutrients as soluble organic and inorganic compounds in aquatic ecosystems has generally been attributed to bacterial action, other mechanisms of nutrient mineralization may be of equal or greater importance. Johannes (1968) stated "... it has been demonstrated beyond reasonable doubt that most of the nitrogen and phosphorus incorporated into aquatic plants and animals is usually regenerated by processes other than direct bacterial action".

The quantity of nutrients released into the water during bacterial growth will depend on the balance between carbon and nutrients essential for the metabolism of bacteria contained in the substrate (Johannes, 1968). If minerals are not present in the substrate in excess of bacterial requirements, they may be obtained from the surrounding water, or if they are not available, growth of bacteria may be limited. In the latter case, any nutrients present will tend to be bound within the bacteria and there is likely to be little release of soluble nutrients into the water. Under such conditions, other methods of releasing nutrients from organisms may be more important in maintaining nutrient levels in a lake. If bacteria die, soluble nutrients may be leached out of the cells soon after death, and the rate of nutrient release will be increased if the cells lyse. This mechanism may also be responsible for some release of nutrients from dead aquatic plants and animals. Johannes (1968) considered that between 25% and 75% of the recycling that occurred in the presence of microorganisms, was brought about by

this process. Golterman (1960, 1964) studied the liberation of minerals from cells of the alga Scenedesmus obliquus which had been killed by u.v. radiation or saturation with chloroform. The alga was in sterile culture. He found that 50% of all phosphorus compounds present in the algae appeared in the water in a few hours. However, less than 20% of the nitrogen was released in the same period. Soluble nitrogen compounds made up only a small percentage of the nitrogen in the cells.

Nutrients released in these ways may be immediately available to other organisms, thus avoiding an intermediate step when the compounds are assimilated by bacteria. Nutrients available to algae and aquatic plants may also be excreted directly from zooplankton. These animals concentrate the nutrients in the particulate matter they consume. Any nutrients in excess of their requirements will be excreted.

The quantity of nutrients released from metabolizing bacteria and zooplankton is, thus, influenced by the concentration of nutrients essential to these organisms in the substrate and the environment. The concentration of nutrients brought in through the inlets of a lake will influence the amount of nutrients in the lake and may therefore be a major factor controlling the mineralization of nutrients by bacteria, apart from being one source of additional nutrients for the primary producers of the lake. The relative importance of autolysis as a means of releasing nutrients will depend on the ability of bacteria to recycle nutrients remaining in the dead organisms after any leaching has occurred.

12.2 POSSIBLE SOURCES OF MINERALS AND TRACE ELEMENTS IN LAKE GRASMERE

Bacterial decomposition in lake water

As noted on p. 338, little response by planktonic bacteria to any addition of organic matter in the form of dead plankton, fragments of Elodea, or nutrients released by healthy shoots of Elodea or by living plankton could be detected during this study. There was some response to decreasing zooplankton numbers and, in the autumn and winter months, larger numbers of bacteria were found over the Elodea beds than in the open water, but it appeared that only a small proportion of the nutrients in Elodea and plankton were being released by bacterial action in the lake water. The possible contribution of the epiphytic bacteria observed on some algae is considered on p. 342.

Bacterial decomposition on Elodea canadensis

The numbers of bacteria in the samples of Elodea fluctuated seasonally (Chapter 6). These fluctuations were attributed primarily to the changing proportions of young and old shoots in the samples. The study of individual leaves showed that as the leaves became moribund the epiphytic bacterial population increased in numbers and a larger proportion of these bacteria was metabolizing. It seemed likely that one supply of nutrients for the Elodea, and for the epiphytic algae on the leaves, was obtained from the rapid degradation of moribund leaves by epiphytic bacteria and also from their recycling of any compounds released from

the healthy leaves and the algae. Any nutrients released by these bacteria appeared to be quickly utilized, presumably by the plant and the epiphytic algae, as the bacterial population of the water over the Elodea was not usually larger than the open water population.

Bacterial decomposition on plankton

The culture experiments described in Chapter 7 indicated that few if any of the bacteria, usually cultured in this study, were directly associated with live phytoplankton or zooplankton. However, the direct observations reported in Chapter 8 showed that the algae sometimes had a conspicuous bacterial flora. In the case of Melosira only moribund cells were colonized, but healthy and moribund cells of Asterionella and Fragilaria were observed, at one time during the study, to have an epiphytic flora. The epiphytes of Melosira may have been actively involved in the degradation of these algae but a similar flora was not seen on moribund cells of other diatoms. The epiphytes of Asterionella and Fragilaria may have obtained nutrients from the healthy and the moribund algae but it is unlikely that these bacteria were essential in nutrient recycling as they were not often present on these algae. These bacteria can be contrasted with the epiphytic bacteria of Elodea which appeared to be very active in the recycling of nutrients.

Excretion from zooplankton

Nutrients excreted from zooplankton will consist mainly of those in excess of the requirements of the animal. In Lake Grasmere no marked increase in the bacterial population occurred as numbers of zooplankton increased but such a response could have been masked by grazing of the bacteria by zooplankton. However, Lake Grasmere does not support large populations of zooplankton and it seems likely that most of the nutrients in the particles assimilated by the animals are stored in their bodies, and that the main release of nutrients from zooplankton occurs on their death.

Autolysis and leaching of organisms

Autolysis and leaching of organisms, which have recently died, might be the reasons why little bacterial response to the death of phytoplankton was detected and the response at the end of zooplankton blooms was not very marked. It can be envisaged that, because leaching occurs, the remaining parts of the plankton are not readily attacked by bacteria as certain essential nutrients are lacking. Without any experimentation on the quantities of nutrients leached from dead organisms, it is impossible to determine whether soluble nutrients thus released are soon lost to bacteria because they are taken up by the plankton, or whether the main effect may be to limit bacterial decomposition because the nutrients become diluted by the water around the organism.

Bacterial decomposition in the mud

Direct examination of mud samples showed that much of the detritus was free of bacteria (Chapter 9). In one sample of surface mud, the numbers of bacteria were smaller than those found in mud, sampled at the same time, from about 5 cm below the surface. Immediate increases in the numbers of bacteria in the mud, following plankton blooms, were not usually detected. However, on one occasion, after a measureable amount of phosphate had been detected in the lake water, the benthic population increased considerably, and there was a moderate increase after heavy rain had resulted in an inflow of silt and attached bacteria to the lake. This latter peak in the bacterial population of the mud might have been an indication of an increased rate of decomposition in the mud or it might have been due primarily to the sedimentation of bacteria on to the mud. It appeared that, in general, the numbers of bacteria in the mud were not large and that the bacterial population of the surface mud was not responding to freshly sedimented organic matter. A large proportion of the nutrients, which were mineralized only slowly by bacteria, must have been buried and lost to short-term circulation. However, with certain combinations of conditions, such as increased temperature and a supply of minerals which had been limiting bacterial growth, bacterial decomposition in the mud probably resulted in a significant release of nutrients into the water.

Some of the nutrients in the subsurface layers of the mud may have been returned to the water later through uptake

by the roots of Elodea. Uptake of phosphorus from reduced subsurface sediments has been demonstrated for Spartina alterniflora (Pomeroy et al., 1969) and Zostera marina L. (McRoy, Barsdate and Nebert, 1972). The role of Elodea roots in the uptake of minerals is controversial, but recently Bristow and Whitcombe (1971) have shown that the roots of Elodea densa can assimilate phosphate. Such an uptake would ensure a more efficient recycling of nutrients.

Inflow from outside the lake

The data presented in Chapter 4 suggested that rainfall, and thus inflow of nutrients and bacteria, in the 7 days before sampling rarely had a direct effect on the bacterial population of the main body of the lake. However, a low level of rainfall in the previous winter, combined with little rain before sampling appeared to limit the size of the bacterial population in the lake in the following autumn. This suggested that a continual supply of nutrients from the inlets was essential if bacterial decomposition was to occur rapidly.

The main nutrients brought into the lake through the inlets were probably trace elements and certain minerals, such as phosphorus, which were normally limiting bacterial growth. The numbers of bacteria over the harbour spring inflow were of the same order of magnitude as the populations in the main body of the lake, which suggested that the quantity of organic matter available for decomposition was

similar to the rest of the lake and that little was brought in through the spring.

The significant correlation between the numbers of bacteria over the harbour spring and rainfall, the limiting effect of low rainfall over a long period on the numbers of bacteria in the main body of the lake and, also, the marked increase in the bacterial population of the mud possibly caused by an addition of phosphate to the lake, all suggested that an inflow of certain minerals and trace elements from outside the lake was an important source of nutrients for the lake organisms.

12.3 CONCLUSIONS

The role of bacteria in the maintenance of nutrient levels in Lake Grasmere

Bacterial degradation often appeared to be limited in Lake Grasmere and the activity of bacteria appeared to be of less importance than the inflow of nutrients and autolysis and leaching of organisms in ensuring a supply of nutrients for the phytoplankton. However, the size of the bacterial population on Elodea suggested that these bacteria were an important link in the recycling of nutrients bound in this plant. Although the benthic bacteria did not seem to rapidly decompose any organic matter which reached the mud, nutrients which were only slowly mineralized might have been available at a later date to aquatic plants, if they could take up these nutrients through their roots.

Therefore, the answer to question (i) on p.338 appeared to be that sufficient nutrients for the primary producers were supplied by a combination of autolysis of organisms, some bacterial decomposition, inflow from outside the lake and possibly uptake of nutrients from the sediment by the rooted vegetation. While bacteria were involved in recycling nutrients, there did not appear to be a simple relationship between the trophic status of the lake and the activity of bacteria.

On the basis of data on bacterial and total plankton respiration in a mesotrophic lake, Tezuka (1970) also concluded that bacteria in water played only a minor role in the mineralization of organic matter. However, this conclusion seems surprising as a bacterial population of usually between 10^4 and 10^5 bacteria per ml was found during a 10 month period in this mesotrophic lake. This population is much larger than the population found in the water of Lake Grasmere and it would be expected that such large numbers of bacteria would contribute significantly to the recycling of nutrients.

The amount of Elodea growing in Lake Grasmere and the size of the phytoplankton populations suggested that the nutrient status of the lake was not low, but bacterial activity appeared often to be limited in the water and in the mud. These observations led to the formulation of question (ii) on p. 338 , the answer to which can only be suggested.

One factor which might have been limiting bacterial activity in the water of Lake Grasmere, which was considered

on p. 343, was that nutrients were being rapidly leached from organisms when they died so that the remaining parts of the organisms lacked certain nutrients essential for bacterial metabolism. If the concentration of the nutrients in the water was too low for bacterial assimilation, little decomposition would then occur.

Bacterial growth might be limited further through competition for minerals and trace elements with organisms, such as Elodea and phytoplankton, especially if these organisms were able to store higher concentrations of nutrients in their cells than immediately required for their metabolism. This has been observed for the aquatic plants Justicia americana and Alternanthera philoxeroides (Boyd, 1969), and the diatom Asterionella formosa (Mackereth, 1953).

Mechanisms, such as these, may have been limiting bacterial activity in Lake Grasmere, but there can be no doubt that some bacterial decomposition of organic matter occurred in the lake water, much occurred on Elodea canadensis and, in the mud, although mineralization of nutrients was usually slow, the remaining fragments of organisms would gradually have been decomposed by the benthic bacteria.

Suggestions for future research

The present study showed that bacteria often do not respond to additional organic matter as might be expected. However, there were still sufficient nutrients for the primary producers in Lake Grasmere. In a lake, the uptake of nutrients from lower levels in the mud by the roots of aquatic plants may be just sufficient to balance the nutrients sedimented on to the mud or may, in some cases, be

one cause of an increased concentration of nutrients in the lake water. This is most likely to occur if the rooted vegetation has been established in the lake for only a relatively short period of time. An investigation of such an uptake of nutrients would be interesting.

The experiments on the bacteria of individual Elodea leaves suggested that an inhibitor might be present in moribund leaves, which was released when the sample was macerated and reduced the numbers of bacteria obtained by the pour plate method. This possibility should be tested before any further plate counts of the bacteria on Elodea are carried out.

The identity and role of the bacteria occasionally seen attached to algal cells were not elucidated in this study, but further work might show that these bacteria are involved in recycling nutrients in algae. More research on the mechanism of bacterial attachment to algae would also be interesting.

The data on Cytophaga showed that the presence of this bacterium may be an indication of increased nutrient levels in a lake, but more work is needed to determine the nature of the nutrients which may be controlling its predominance.

Research into the kinds of nutrients leached from plankton when they die and the rate at which they are liberated, and experiments on the effect of certain nutrients on the rate of bacterial decomposition of plankton, which has been leached for different lengths of time, might help to determine which nutrients were limiting bacterial decomposition in Lake Grasmere. This would help to clarify

the relationships between bacterial activity and nutrient concentrations in this lake and other lakes.

It is clear from this study that more work is needed on bacteria, such as those seen on algae, which are not readily cultured but may be involved in decomposition of organisms and, also, on the factors controlling the activity of aquatic bacteria in general.

ACKNOWLEDGMENTS

This work was carried out in the Botany Department of the University of Canterbury and I wish to thank Professor W.R. Philipson for making available facilities and funds, and for the use of the Field Station at Cass during field trips. I would also like to express my gratitude to Dr J.D. Allen for his supervision and encouragement throughout this project.

I wish to thank Dr V.M. Stout for help with the field work, chemical data and data on zooplankton and for valuable criticism of the manuscript. Dr J.S. Waid suggested the application of autoradiography to this work and I wish to thank him for encouragement during the development of the technique and criticism of this section of the thesis. I also thank Dr E.A. Flint for identifying the algae and for helpful discussions on algae.

I would also like to thank Mrs H.J. Langer and Mr A.R.D. Ramsay for advice and assistance in the statistical analysis, Mr F. McGregor for assistance with photography, Miss S. Bullock for advice and help in the use of the electron microscope and the Zoology Department for making the water sampler.

REFERENCES

ALLEN H.L. (1969) Chemo-organotrophic utilization of dissolved organic compounds by planktic algae and bacteria in a pond. Int. Revue ges. Hydrobiol. Hydrogr. 54, 1-33.

ALLEN H.L. (1971) Primary productivity, chemo-organotrophy, and nutritional interactions of epiphytic algae and bacteria on macophytes in the littoral of a lake. Ecol. Monogr. 41, 97-127.

ALTSCHULER S.J. and RILEY G.A. (1967) Microbiological studies in Long Island Sound. Bull. Bingham oceanogr. Coll. 19, 81-88.

AMERICAN PUBLIC HEALTH ASSOCIATION (1965) Standard Methods for Examination of Water and Wastewater Including Bottom Sediments and Sludges, 12th ed. American Public Health Association Inc., New York.

ANDERSON J.G. and MEADOWS P.S. (1969) Bacteria on intertidal sand grains. Hydrobiologia 33, 33-46.

ANDERSON R.L. and ORDAL E.J. (1961) Cytophaga succinicans sp.n., a facultatively anaerobic, aquatic myxobacterium. J. Bact. 81, 130-138.

ANTHONY C. and ZATMAN L.J. (1964) The microbial oxidation of methanol. 1. Isolation and properties of Pseudomonas sp. M 27. Biochem. J. 92, 609-614.

ANTHONY E.H. (1970) Bacteria in estuarine (Bras d'Or Lake) sediment. Can. J. Microbiol. 16, 373-389.

ANTHONY E.H. and HAYES F.R. (1964) Lake water and sediment. VII. Chemical and optical properties of water in relation to the bacterial counts in the sediments of twenty-five North American lakes. Limnol. Oceanogr. 9, 35-41.

BADDELEY M.S. (1971) Biochemical aspects of senescence. In Ecology of Leaf Surface Micro-organisms (T.F. Preece and C.H. Dickinson, eds.). Academic Press, London. 415-429.

BARRITT M.M. (1936) The intensification of the Voges-Proskauer reaction by the addition of α-naphthol. J. Path. Bact. 42, 441.

BATOOSINGH E. and ANTHONY E.H. (1971) Direct and indirect observations of bacteria on marine pebbles. Can. J. Microbiol. 17, 655-664.

BÉLANGER L.F. (1961) Staining processed radioautographs. Stain Techn. 36, 313-317.

BELL G.R., HOSKINS G.E. and HODGKISS W. (1971) Aspects of the characterization, identification, and ecology of the bacterial flora associated with the surface of stream-incubating Pacific salmon (Oncorhynchus) eggs. J. Fish. Res. Bd. Canada 28, 1511-1525.

BERLIN M. and RYLANDER R. (1963) Autoradiographic detection of radioactive bacteria introduced into sea water and sewage. J. Hyg., Camb. 61, 307-315.

BHAT J.V. and BARKER H.A. (1948) Studies on a new oxalate-decomposing bacterium, Vibrio oxaliticus. J. Bact. 55, 359-368.

BOYD C.E. (1969) Production, mineral nutrient absorption, and biochemical assimilation by Justicia americana and Alternanthera philoxeroides. Arch. Hydrobiol. 66, 139-160.

BREED R.S., MURRAY E.G.D. and SMITH N.R. (1957) Bergey's Manual of Determinative Bacteriology, 7th ed. Williams and Wilkins, Baltimore.

BRISTOW J.M. and WHITCOMBE M. (1971) The role of roots in the nutrition of aquatic vascular plants. Amer. J. Bot. 58, 8-13.

BROCK M.L. and BROCK T.D. (1968) The application of micro-autoradiographic techniques to ecological studies. Mitt. int. Ver. Limnol. 15, 1-29.

BROCK T.D. and BROCK M.L. (1966) Autoradiography as a tool in microbial ecology. Nature, Lond. 209, 734-736.

BUCK J.D. and CLEVERDON R.C. (1960) The spread plate as a method for the enumeration of marine bacteria. Limnol. Oceanogr. 5, 78-80.

BURGES A. (1965) The soil microflora - its nature and biology. In Ecology of Soil-borne Plant Pathogens (K.F. Baker and W.C. Snyder, eds.). University of California Press. 21-32.

CARLUCCI A.F. and PRAMER D. (1957) Factors influencing the plate method for determining abundance of bacteria in sea water. Proc. Soc. exp. Biol., N.Y. 96, 392-394.

CARO L.G. and van TUBERGEN R.P. (1962) High resolution autoradiography. 1. Methods. J. Cell. Biol. 15, 173-188.

CARPENTER K.P., LAPAGE S.P. and STEEL K.J. (1966) Biochemical identification of Enterobacteriaceae. In Identification Methods for Microbiologists, Part A (B.M. Gibbs and F.A. Skinner, eds.). Academic Press, London. 21-33.

CARPENTER P.L. (1939) Bacterial counts in the muds of Crystal lake - an oligotrophic lake of northern Wisconsin. J. sed. Petrol. 9, 3-7.

CHAN E.C.S. and McMANUS E.A. (1967) Development of a method for the total count of marine bacteria on algae. Can. J. Microbiol. 13, 295-301.

CHAN E.C.S. and McMANUS E.A. (1969) Distribution, characterization and nutrition of marine microorganisms from the algae Polysiphonia lanosa and Ascophyllum nodosum. Can. J. Microbiol. 15, 409-420.

CHEN M. (1968) Ecological studies of bacteria in the water of Tuttle Creek Reservoir. Kans. State Univ. Diss. Abstr. 29B, 1917.

CHU, S.P. (1942) The influence of the mineral composition of the medium on the growth of planktonic algae. 1. Methods and culture media. J. Ecol. 30, 284-325.

CLARK D.S. (1971) Studies on the surface plate method of counting bacteria. Can. J. Microbiol. 17, 943-946.

COLER R.A. and GUNNER H.B. (1969) The rhizosphere of an aquatic plant (Lemna minor). Can. J. Microbiol. 15, 964-966.

COLLINS V.G. (1957) Planktonic bacteria. J. gen. Microbiol. 16, 268-272.

COLLINS V.G. (1960) The distribution and ecology of Gram-negative organisms other than Enterobacteriaceae in lakes. J. appl. Bact. 23, 510-514.

COLLINS V.G. (1963) The distribution and ecology of bacteria in freshwater. Proc. Soc. Wat. Treat. Exam. 12, 40-73.

COLLINS V.G. (1970) Recent studies of bacterial pathogens of freshwater fish. Wat. Treatment Exam. 19, 3-31.

COLLINS V.G. and WILLOUGHBY L.G. (1962) The distribution of bacteria and fungal spores in Blelham Tarn with particular reference to an experimental overturn. Arch. Mikrobiol. 43, 294-307.

COLWELL R.R. (1969) Numerical taxonomy of the flexibacteria. J. gen. Microbiol. 58, 207-215.

COLWELL R.R., CITARELLA R.V. and CHEN P.K. (1966) DNA base composition of Cytophaga marinoflavum n.sp. determined by buoyant density measurements in cesium chloride. Can. J. Microbiol. 12, 1099-1103.

COOPER B.A., MURRAY E.G.D. and KLEEREKOPER H. (1953) The bottom sediments of Lake Lauzon, Montcalm County, Province of Quebec. II. Bacteriological investigations. Rev. Can. Biol. 12, 457-494.

COWAN S.T. (1968) A Dictionary of Microbial Taxonomic Usage. Oliver and Boyd, Edinburgh.

COWELL N.D. and MORISETTI M.D. (1969) Microbiological techniques - some statistical aspects. J. Sci. Fd. Agric. 20, 573-579.

DAFT M.J. and STEWART W.D.P. (1971) Bacterial pathogens of freshwater blue-green algae. New Phytol. 70, 819-829.

DAVIS B.D., DULBECCO R., EISEN H.W., GINSBERG H.S. and WOOD W.B. (1969) Microbiology, International ed. Harper and Row, New York.

DRABKOVA V.G. (1965) Dynamics of the bacterial number, generation time, and production of bacteria in the water of Red Lake (Punnus-Jarvi). Mikrobiologiya 34, 933-938.

DROOP M.R. and ELSON K.G.R. (1966) Are pelagic diatoms free from bacteria? Nature, Lond. 211, 1096-1097.

DUFF D.C.B., BRUCE D.L. and ANTIA N.J. (1966) The antibacterial activity of marine planktonic algae. Can. J. Microbiol. 12, 877-884.

DUGAN P.R. (1972) Biochemical Ecology of Water Pollution. Plenum Press, N.Y. 140.

DWORKIN M. (1966) Biology of the myxobacteria. A. Rev. Microbiol. 20, 75-106.

DWORKIN M. and FOSTER J.W. (1956) Studies on Pseudomonas methanica (Söhngen) nov. comb. J. Bact. 72, 646-659.

DYE D.W. (1962) The inadequacy of the usual determinative tests for the identification of Xanthomonas spp. N.Z. J. Sci. 5, 393-416.

EISENHART C. and WILSON P.W. (1943) Statistical methods and control in bacteriology. Bact. Rev. 7, 57-137.

EKLUND C. and LANKFORD C.E. (1967) Laboratory Manual for General Microbiology. Prentice-Hall, New Jersey.

FAGER E.W. (1969) Recurrent group analysis in the classification of flexibacteria. J. gen. Microbiol. 58, 179-187.

FANGMAN W.L. (1969) Specificity and efficiency of thymidine incorporation in Escherichia coli lacking thymidine phosphorylase. J. Bact. 99, 681-687.

FISHER R.A., THORNTON H.G. and MACKENZIE W.A. (1922) The accuracy of the plating method of estimating the density of bacterial populations. Ann. appl. Biol. 9, 325-359.

FOGG G.E. (1971) Extracellular products of algae in freshwater. Arch. Hydrobiol. Beih. Ergebn. Limnol. 5, 1-25.

FOLLETT E.A.C. and WEBLEY D.M. (1965) An electron microscope study of the cell surface of Cytophaga johnsonii and some observations on related organisms. Antonie van Leeuwenhoek 31, 361-382.

FONDÉN R. (1968) A yeast-extract peptone agar for the determination of heterotrophic bacteria in lakes. Vatten 24, 161-166.

FONDÉN R. (1969a) Heterotrophic bacteria in Lake Mälaren and Lake Hjälmaren. Oikos 20, 344-372.

FONDÉN R. (1969b) Nutritional classification of some limnic bacterial populations. Oikos 20, 373-383.

FOX D.L. and LEWIN R.A. (1963) A preliminary study of the carotenoids of some flexibacteria. Can. J. Microbiol. 9, 753-768.

FRED E.B., WILSON F.C. and DAVENPORT A. (1924) The distribution and significance of bacteria in Lake Mendota. Ecology 5, 322-339.

GERLETTI M. and MELCHIORRI-SANTOLINI U. (1968) A comparative study on primary productivity, bacterial microflora and ecological factors in lakes: Bolsena, Maggiore, Mergozzo and Monate. Mem. Ist. Ital. Idrobiol. 24, 161-195.

GOLDMAN C.R., GERLETTI M., JAVORNICKÝ P., MELCHIORRI-SANTOLINI U. and de AMEZAGA E. (1968) Primary productivity, bacteria, phyto- and zooplankton in Lake Maggiore: correlations and relationships with ecological factors. Mem. Ist. Ital. Idrobiol. 23, 49-127.

GOLTERMAN H.L. (1960) Studies on the cycle of elements in fresh water. Acta bot. neerl. 9, 1-58.

GOLTERMAN H.L. (1964) Mineralization of algae under sterile conditions by bacterial breakdown. Verh. int. Ver. Limnol. 15, 544-548.

GOLTERMAN H.L. (1968) Investigations on photosynthesis and mineralization in experimental ponds. Mitt. int. Ver. Limnol. 14, 25-30.

GRÄF W. and STÜRZENHOFECKER P. (1965) Myxobakterientypenquotient als Eutrophierungsindikator bei Oberflächengewässern. Arch. Hyg. Bakt. 149, 265-273.

GRAHAM V.E. and YOUNG R.T. (1934) A bacteriological study of Flathead Lake, Montana. Ecology 15, 101-109.

GRAY E. (1951) The ecology of the bacteria of Hobson's Brook, a Cambridgeshire chalk stream. J. gen. Microbiol. 5, 840-859.

GRIVELL A.R. and JACKSON J.F. (1968) Thymidine kinase: evidence for its absence from Neurospora crassa and some other micro-organisms, and the relevance of this to the specific labelling of deoxyribonucleic acid. J. gen. Microbiol. 54, 307-317.

GUDE W.D. (1968) Autoradiographic Techniques. Prentice-Hall, New Jersey.

GUNKEL W., JONES G.E. and ZOBELL C.E. (1961) Influence of volume of nutrient agar medium on development of colonies of marine bacteria. Helgoländer wiss. Meeresunters. 8, 85-93.

GUTHRIE R.K. (1968) Bacterial cycles and water quality. SW. Wat. Wks J. 50, 54-56.

HAGEDORN H. (1969) Die vertikale Verteilung von Thiamin, Bakterien und Phytoplankton in drei ostholsteinischen Seen. Ber. dt. bot. Ges. 82, 223-234.

HALE C.M.F. (1953) The use of phosphomolybdic acid in the mordanting of bacterial cell walls. Lab. Pract. 2, 115-116.

HARRIGAN W.F. and McCance M.E. (1966) Laboratory Methods in Microbiology. Academic Press, London.

HARRINGTON A.A. and KALLIO R.E. (1960) Oxidation of methanol and formaldehyde by Pseudomonas methanica. Can. J. Microbiol. 6, 1-7.

HARRIS D.O. (1971) Growth inhibitors produced by the green algae (Volvocaceae). Arch. Mikrobiol. 76, 47-50.

HAYES F.R. and ANTHONY E.H. (1959) Lake water and sediment. VI. The standing crop of bacteria in lake sediments an its place in the classification of lakes. Limnol. Oceanogr. 4, 299-315.

HAYES F.R. and PHILLIPS J.E. (1958) Lake water and sediment. IV. Radiophosphorus equilibrium with mud, plants and bacteria under oxidized and reduced conditions. Limnol. Oceanogr. 3, 459-475.

HAYES P.R. (1963) Studies on marine flavobacteria. J. gen. Microbiol. 30, 1-19.

HAYWARD A.C. (1960) Relationship between Protoaminobacter ruber and some red-pigmented pseudomonads. J. appl. Bact. 23, xii.

HAYWARD J.A. (1967) The Waimakariri Catchment. Tussock Grasslands and Mountain Lands Inst., Special Publ. No. 5.

HENDRIE M.S., HODGKISS W. and SHEWAN J.M. (1964) Considerations on organisms of the Achromobacter-Alcaligenes group. Annls Inst. Pasteur Lille 15, 43-59.

HENDRIE M.S., MITCHELL T.G. and SHEWAN J.M. (1968) The identification of yellow-pigmented rods. In Identification Methods for Microbiologists, Part B (B.M. Gibbs and D.A. Shapton, eds.). Academic Press, London. 67-78.

HENRICI A.T. (1938) Studies of freshwater bacteria. IV. Seasonal fluctuations of lake bacteria in relation to plankton production. J. Bact. 35, 129-139.

HENRICI A.T. AND McCOY E. (1938) The distribution of heterotrophic bacteria in the bottom deposits of some lakes. Trans. Wisc. Acad. Sci. Arts Lett. 31, 323-361.

HIRSCH P. (1968) Gestielte und Knospende Bakterien: Spezialisten für C-1 Stoffwechsel an nährstoffarmen Standorten. Mitt. int. Ver. Limnol. 14, 52-63.

HOBBIE J.E. and WRIGHT R.T. (1965) Bioassay with bacterial uptake kinetics: glucose in freshwater. Limnol. Oceanogr. 10, 471-474.

HODGKISS W. and SHEWAN J.M. (1968) Problems and modern principles in the taxonomy of marine bacteria. In Advances in Microbiology of the Sea (M.R. Droop and E.J. Ferguson Wood, eds.). Academic Press, London. 1, 127-166.

HOLM-HANSEN O. and BOOTH C.R. (1966) The measurement of adenosine triphosphate in the ocean and its ecological significance. Limnol. Oceanogr. 11, 510-519.

HORNE R.W. (1965) Negative staining methods. In Techniques for Electron Microscopy, 2nd ed. (D.H.Kay, ed.). Blackwell, Oxford.

HUGH R. and LEIFSON E. (1953) The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. J. Bact. 66, 24-26.

HUGHES J.C. and LUND J.W.G. (1962) The rate of growth of Asterionella formosa Hass. in relation to its ecology. Arch. Mikrobiol. 42, 117-129.

IRWIN J. (1969) Lake Grasmere, Provisional Bathymetry 1:3168. N.Z. Oceanogr. Inst. Chart, Lake series

JANNASCH H.W. (1956) Vergleichende bakteriologische Untersuchung der Adsorptionswirkung des Nil-Treibschlammes. Ber. limnol. Flussstn Freudenthal 7, 21-27. (cited by Wuhrmann, 1964).

JANNASCH H.W. (1958) Studies on planktonic bacteria by means of a direct membrane filter method. J. gen. Microbiol. 18, 609-620.

JANNASCH H.W. (1965) Biological significance of bacterial counts in aquatic environments. Coll. Repr. Woods Hole Oceanogr. Inst. No. 1490.

JANNASCH H.W. and JONES G.E. (1959) Bacterial populations in sea water as determined by different methods of enumeration. Limnol. Oceanogr. 4, 128-139.

JENSEN V. (1971) The bacterial flora of beech leaves. In Ecology of Leaf Surface Micro-organisms (T.F. Preece and C.H. Dickinson, eds.). Academic Press, London. 463-469.

JOHANNES R.E. (1965) Influence of marine protozoa on nutrient regeneration. Limnol. Oceanogr. 10, 434-442.

JOHANNES R.E. (1968) Nutrient regeneration in lakes and oceans. In Advances in Microbiology of the Sea (M.R. Droop and E.J. Ferguson Wood, eds.). Academic Press, London. 1, 203-213.

JONES J.G. (1970) Studies on freshwater bacteria: effect of medium composition and method on estimates of bacterial population. J. appl. Bact. 33, 679-686.

JONES J.G. (1971) Studies on freshwater bacteria: factors which influence the population and its activity. J. Ecol. 59, 593-613.

KANEDA T. and ROXBURGH J.M. (1959) A methanol-utilizing bacterium. I. Description and nutritional requirements. Can. J. Microbiol. 5, 87-98.

KING E.O., WARD M.K. and RANEY D.E. (1954) Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. clin. Med. 44, 301-307.

KLINCĀRE A.A., KRĒSLINA D.J. and MISHKE I.V. (1971) Composition and activity of the epiphytic microflora of some agricultural plants. In Ecology of Leaf Surface Micro-organisms (T.F. Preece and C.H. Dickinson, eds.). Academic Press, London. 191-201.

KORNFELD S. and GLASER L. (1960) The enzymic synthesis of thymidine diphosphate glucose and thymidine diphosphate rhamnose. Biochim. Biophys. Acta 42, 548-550.

KOVACS N. (1956) Identification of Pseudomonas pyocyanea by the oxidase reaction. Nature, Lond. 178, 703.

KROULIK J.T., BURKEY L.A. and WISEMAN H.G. (1955) The microbial populations of the green plant and of the cut forage prior to ensiling. J. Dairy Sci. 38, 256-262.

KUZNETSOV S.I. (1968) Recent studies on the role of microorganisms in the cycling of substances in lakes. Limnol. Oceanogr. 13, 211-224.

LAST F.T. and DEIGHTON F.C. (1965) The non-parasitic microflora on the surfaces of living leaves. Trans. Br. mycol. Soc. 48, 83-99.

LEADBETTER E.R. and FOSTER J.W. (1958) Studies on some methane-utilizing bacteria. Arch. Mikrobiol. 30, 91-118.

LEVINA R.I. (1964) Antagonism between protococci and the coli-typhus bacteria. Mikrobiologiya 33, 787-792.

LEWIN R.A. (1969) A classification of flexibacteria. J. gen. Microbiol. 58, 189-206.

LEWIN R.A. and LOUNSBERY D.M. (1969) Isolation, cultivation and characterization of flexibacteria. J. gen. Microbiol. 58, 145-170.

MACKERETH F.J. (1953) Phosphorus utilization by Asterionella formosa Hass. J. exp. Bot. 4, 296-313.

MANDEL M. and LEWIN R.A. (1969) Deoxyribonucleic acid base composition of flexibacteria. J. gen. Microbiol. 58, 171-178.

Manual of Microbiological Methods (1957) Society of American Bacteriologists. McGraw-Hill, New York.

MARSHALL K.C., STOUT R. and MITCHELL R. (1971) Selective sorption of bacteria from seawater. Can. J. Microbiol. 17, 1413-1416.

McCOY E. and SARLES W.B. (1969) Bacteria in lakes: populations and functional relations. In Eutrophication: Causes, Consequences, Correctives. Nat. Acad. Sci., Washington. 331-339.

McMEEKIN T.A., PATTERSON J.T. and MURRAY J.G. (1971) An initial approach to the taxonomy of some Gram-negative yellow pigmented rods. J. appl. Bact. 34, 699-716.

McROY C.P., BARSDATE R.J. and NEBERT M. (1972) Phosphorus cycling in an eelgrass (Zostera marina L.) ecosystem. Limnol. Oceanogr. 17, 58-67.

MEADOWS P.S. (1971) The attachment of bacteria to solid surfaces. Arch. Mikrobiol. 75, 374-381.

MEYNELL G.G. and MEYNELL E. (1970) Theory and Practice in Experimental Bacteriology, 2nd ed. Cambridge University Press.

MITCHELL T.G., HENDRIE M.S. and SHEWAN J.M. (1969) The taxonomy, differentiation and identification of Cytophaga species. J. appl. Bact. 32, 40-50.

MUNRO A.L.S. and BROCK T.D. (1968) Distinction between bacterial and algal utilization of soluble substances in the sea. J. gen. Microbiol. 51, 35-42.

NAGUIB M. and OVERBECK J. (1970) On methane oxidizing bacteria in fresh waters. I. Introduction to the problem and investigations on the presence of obligate methane oxidizers. Z. allg. Mikrobiol. 10, 17-36.

NAUWERCK A. (1963) Die Beziehungen zwischen Zooplankton und Phytoplankton in See Erken. Symb. bot. upsal. 17, 1-163.

NEDWELL D.B. and FLOODGATE G.D. (1971) The seasonal selection by temperature of heterotrophic bacteria in an intertidal sediment. Mar. Biol. 11, 306-310.

NICHOLSON J.H. (1972) Studies on the phylloplane microflora of Pinus radiata D. Don and its interaction with the fungal pathogen Dothistroma pini Hulbary. Ph. D. Thesis, University of Canterbury.

ODUM H.T. (1957) Trophic structure and productivity of Silver Springs, Florida. Ecol. Monogr. 27, 55-112.

OKAZAKI R. (1959) Isolation of a new deoxyribosidic compound, thymidine diphosphate rhamnose. Biochem. Biophys. Res. Commun. 1, 34-38.

OPPENHEIMER C.H. and VANCE M.H. (1960) Attachments of bacteria to the surfaces of living and dead microorganisms in marine sediments. Z. allg. Mikrobiol. 1, 47-52.

OVERBECK J. (1968) Prinzipielles zum Vorkommen der Bakterien im See. Mitt. int. Ver. Limnol. 14, 134-144.

OVERBECK J. and BABENZIEN H. -D. (1964) Bakterien und Phytoplankton eines Kleingewässers im Jahreszyklus. Z. allg. Mikrobiol. 4, 59-76.

OVERBECK J. and OHLE W. (1964) Contributions to the biology of methane oxidizing bacteria. Verh. int. Ver. Limnol. 15, 535-543.

PAASCHE E. (1960) On the relationship between primary production and standing stock of phytoplankton. J. Cons. perm. int. Explor. Mer 26, 33-48.

PAERL H.W. and GOLDMAN C.R. (1972) Heterotrophic assays in the detection of water masses at Lake Tahoe, California. Limnol. Oceanogr. 17, 145-148.

PARR L.W. (1939) Coliform bacteria. Bact. Rev. 3, 1-48.

PARSONS T.R. and STRICKLAND J.D.H. (1962) On the production of particulate organic carbon by heterotrophic processes in sea water. Deep-Sea Res. 8, 211-222.

PEEL D. and QUAYLE J.R. (1961) Microbial growth on C_1 compounds. 1. Isolation and characterization of Pseudomonas AM 1. Biochem. J. 81, 465-469.

POINDEXTER J.S. (1964) Biological properties and classification of the Caulobacter group. Bact. Rev. 28, 231-295.

POMEROY L.R., JOHANNES R.E., ODUM E.P. and ROFFMAN B. (1969) The phosphorus and zinc cycles and productivity of a salt marsh. In Symposium on Radioecology (D.J. Nelson and F.C. Evans ed.). Proc. 2nd Nat. Symp. Ann Arbor, Michigan.

POTAENKO Y.S. (1968) Seasonal dynamics of total bacterial number and biomass in water of Narochan Lakes. Mikrobiologiya 37, 441-446.

POTAENKO Y.S. and MIKHEEVA T.M. (1969) Relationships between bacteria and phytoplankton. Mikrobiologiya 38, 603-607.

POTTER L.F. (1964) Planktonic and benthic bacteria of lakes and ponds. In Principles and Applications in Aquatic Microbiology (H. Heukelekian and N.C. Dondero, eds.). Wiley, New York. 148-166.

POTTER L.F. and BAKER G.E. (1956) The microbiology of Flathead and Rogers Lakes, Montana. I. Preliminary survey of the microbial populations. Ecology 37, 351-355.

POTTER L.F. and BAKER G.E. (1961) The microbiology of Flathead and Rogers Lakes, Montana. II. Vertical distribution of the microbial populations and chemical analyses of their environments. Ecology 42, 338-348.

PRAMER D. and SCHMIDT E.L. (1965) Experimental Soil Microbiology. Burgess, Minneapolis.

PRITCHARD R.H., BARTH P.T. and COLLINS J. (1969) Control of DNA synthesis in bacteria. In Microbial Growth, 19th Symposium of Society for General Microbiology. Cambridge University Press. 263-297.

RACHMELER M., GERHART J. and ROSNER J. (1961) Limited thymidine uptake in Escherichia coli due to an inducible thymidine phosphorylase. Biochem. Biophys. Acta 49, 222-225.

REUSZER H.W. (1933) Marine bacteria and their role in the cycle of life in the sea. III. The distribution of bacteria in the ocean waters and muds about Cape Cod. Biol. Bull. 65, 480-497.

RIBBONS D.W., HARRISON J.E. and WADZINSKI A.M. (1970) Metabolism of single carbon compounds. A. Rev. Microbiol. 24, 135-158.

RIGOMIER D. (1967) Premier bilan de la population bactérienne hétérotrophe du zooplancton. C. r. Séanc. Soc. Biol. 161, 679-683.

RODINA A.G. (1968) Microorganisms of an acidotrophic lake. Mikrobiologiya 37, 129-134.

RODINA A.G. (1969) Bacterial population of humified lakes. Mikrobiologiya 38, 446-451.

RODINA A.G. and KUZ'MITSKAYA N.K. (1963) Abundance and distribution of bacterioplankton in Lake Ladoga. Mikrobiologiya 32, 248-253.

RODINA A.G. and KUZ'MITSKAYA N.K. (1964) The species composition of the heterotrophic microorganisms in the water-mass of Lake Ladoga. Mikrobiologiya 33, 894-900.

ROGERS A.W. (1967) Techniques of Autoradiography. Elsevier, Amsterdam.

RUSCHKE R. (1968) Die Bedeutung von Wassermyxobakterien für den Abbau organischen Materials. Mitt. int. Ver. Limnol. 14, 164-167.

RUSCHKE R. and RATH M. (1965) Sporocytophaga cauliformis Knorr und Gräf, eine Myxobakterienart mit grosser Bedeutung für den Abbau organischen Materials. Arch. Hydrobiol./Suppl. 28, 377-402.

RUTTNER F. (1959) Einige Beobachtungen über das Phytoplankton Nord-Italienischer Seen. Mem. Ist. Ital. Idrobiol. 11, 73-111.

SAUNDERS G.W. Jr. (1959) The application of radioactive tracers to the study of lake metabolism. Ph.D. Thesis, University of Michigan. University Microfilms, Inc., Ann Arbor, Michigan. Mic 59-2175.

SCHEGG E. (1968) Beziehungen zwischen Planktonentwicklung und Bakterien im Vierwaldstättersee und Rotsee. Schweiz. Z. Hydrol. 30, 289-296.

SCHEGG E. and RUSCHKE R. (1970) Beitrag zur Bakterienökologie einiger Seen des Schwarzwaldes und der Schweiz. Schweiz. Z. Hydrol. 32, 192-213.

SEKI H. (1967) Ecological studies on the lipolytic activity of microorganisms in the sea of Aburatsubo Inlet. Rec. oceanogr. Wks Japan 9, 75-113.

SEKI H., SKELDING J. and PARSONS T.R. (1968) Observations on the decomposition of a marine sediment. Limnol. Oceanogr. 13, 440-447.

SHEWAN J.M. (1963) The differentiation of certain genera of Gram negative bacteria frequently encounted in marine environments. In Symposium on Marine Microbiology (C.H. Oppenheimer, ed.). Thomas, Springfield. 499-521.

SHEWAN J.M., HOBBS G. and HODGKISS W. (1960) A determinative scheme for the identification of certain genera of Gram-negative bacteria, with special reference to the Pseudomonadaceae. J. appl. Bact. 23, 379-390.

SHILO M. (1971) Biological agents which cause lysis of blue-green algae. Mitt. int. Ver. Limnol. 19, 206-213.

SHUL'GOVSKAYA E.M., ANDREEVA E.A. and RABOTNOVA I.L. (1971) Isolation and properties of a methanol-oxidizing bacterium Pseudomonas methanolica n.sp. Mikrobiologiya 40, 752-754.

SIEBURTH J. McN. (1959) Antibacterial activity of Antarctic marine phytoplankton. Limnol. Oceanogr. 4, 419-424.

SIEBURTH J. McN. (1968) The influence of algal antibiosis on the ecology of marine microorganisms. In Advances in Microbiology of the sea (M.R. Droop and E.J. Ferguson Wood, eds.). Academic Press, London. 1, 63-94.

SIEBURTH J. McN. (1971) Distribution and activity of oceanic bacteria. Deep-Sea Res. 18, 1111-1121.

SILVEY J.K.G. and ROACH A.W. (1964) Studies on microbiotic cycles in surface waters. J. Am. Wat. Wks Ass. 56, 60-72.

SIMIDU U., ASHINO K. and KANEKO E. (1971) Bacterial flora of phyto- and zooplankton in the inshore water of Japan. Can. J. Microbiol. 17, 1157-1160.

SINHA S. (1971) The microflora on leaves of Capsicum annuum (L.) Watt E.D., Solanum melongena L., Solanum tuberosum L. and Lycopersicum esculentum Mill. In Ecology of Leaf Surface Micro-organisms (T.F. Preece and C.H. Dickinson, eds.). Academic Press, London. 175-189.

SKERMAN V.B.D. (1967) A Guide to the Identification of the Genera of Bacteria, 2nd ed. Williams and Wilkins, Baltimore.

SNEDECOR G.W. and COCHRAN W.G. (1967) Statistical methods, 6th ed. Iowa State University Press, Ames, Iowa.

SNOW L.M. and FRED E.B. (1926) Some characteristics of the bacteria of Lake Mendota. Trans. Wisc. Acad. Sci. Arts Lett. 22, 143-154.

SOKAL R.R. and SNEATH P.H.A. (1963) Principles of Numerical Taxonomy. Freeman, London.

SOROKIN Y.I. (1969) Experimental evaluation of effect of pressure and temperature on metabolism of microflora in deep ocean waters. Mikrobiologiya 38, 739-743.

SOROKIN Y.I. (1970a) Interrelations between sulphur and carbon turnover in leromictic lakes. Arch. Hydrobiol. 66, 391-446.

SOROKIN Y.I. (1970b) Determination of the activity of heterotrophic microflora in the ocean using C^{14}-containing organic matter. Mikrobiologiya 39, 133-138.

SPENCER C.P. (1952) On the use of antibiotics for isolating bacteria-free cultures of marine phytoplankton organisms. J. mar. biol. Ass. U.K. 31, 97-106.

STANIER R.Y. (1940) Studies on the cytophagas. J. Bact. 40, 619.

STANIER R.Y., DOUDEROFF M. and ADELBERG E.A. (1970) General Microbiology. 3rd ed. Macmillan, London.

STARK W.H. and McCOY E. (1938) Distribtuion of bacteria in certain lakes of northern Wisconsin. Zentbl. Bakt. ParasitKde 98, 201-209.

STARK W.H., STADLER J. and McCOY E. (1938) Some factors affecting the bacterial population of freshwater lakes. J. Bact. 36, 653-654.

STARR T.J. and ORDAL E.J. (1953) A study of marine myxobacteria. Tech. Rep. No. 14, Office of Naval Research, U.S.A.

STEEMANN NIELSEN E. (1952) The use of radioactive carbon ^{14}C for measuring organic production in the sea. J. Cons. perm. int. Explor. Mer 18, 117-140.

STEEMANN NIELSEN E. (1955) The production of antibiotics by plankton algae and its effect upon bacterial activities in the sea. Deep-Sea Res. Suppl. to Vol. 3, 281-289.

ŠTĚPÁNEK M. (1968) Verhältnis des Phytoplanktons zur Bakterienbesiedlung der Sedlice-Talsperre. Mitt. int. Ver. Limnol. 14, 201-211.

STOCKS P.K. and McCLESKEY C.S. (1964) Identity of the pink-pigmented methanol-oxidizing bacteria as Vibrio extorquens. J. Bact. 88, 1065-1070.

STOTT M.A. (1971) Studies on the physiology of some leaf saprophytes. In Ecology of Leaf Surface Microorganisms (T.F. Preece and C.H. Dickinson, eds.). Academic Press, London. 203-210.

STOUT J.D. (1960) Bacteria of soil and pasture leaves at Claudelands Showgrounds. N.Z. J. agric. Res. 3, 413-430.

STOUT V.M. (1969) Lakes in the mountain region of Canterbury, New Zealand. Verh. int. Ver. Limnol. 17, 404-413.

STOUT V.M. (1972) Plankton composition in relation to nutrient inflow in a small New Zealand lake. Verh. int. Ver. Limnol. In press.

STRAKA R.P. and STOKES J.L. (1957) Rapid destruction of bacteria in commonly used diluents and its elimination. Appl. Microbiol. 5, 21-25.

STRUGGER S. (1948) Fluorescence microscope examination of bacteria in soil. Can. J. Res. 26, Sec. C. 188-193.

STRZELCZYK E., ANTCZAK E. and KUCHCIŃSKA H. (1971) Studies on morphology, nutritional requirements, biochemical activity and antibiotic resistance of heterotrophic water bacteria. Arch. Hydrobiol. 69, 95-105.

STRZELCZYK E. and MIELCZAREK A. (1971) Comparative studies on metabolic activity of planktonic, benthic and epiphytic bacteria. Hydrobiologia 38, 67-77.

TAKAHASHI M. and ICHIMURA S. (1968) Vertical distribution and organic matter production of photosynthetic sulfur bacteria in Japanese lakes. Limnol. Oceanogr. 13, 644-655.

TAYLOR C.B. (1940) Bacteriology of fresh water. I. Distribution of bacteria in English lakes. J. Hyg., Camb. 40, 616-640.

TAYLOR C.B. (1941) Bacteriology of fresh water. II. The distribution and types of coliform bacteria in lakes and streams. J. Hyg., Camb. 41, 17-38.

TAYLOR C.B. (1942) Bacteriology of fresh water. III. The types of bacteria present in lakes and streams and their relationships to the bacterial flora of soil. J. Hyg., Camb. 42, 284-296.

TAYLOR C.B. (1949) Factors affecting the bacterial population of lake waters. Proc. Soc. appl. Bact. 1, 4-10.

TAYLOR L.R. (1961) Aggregation, variance and the mean. Nature, Lond. 189, 732-735.

TEZUKA Y. (1970) Distribution of heterotrophic bacteria and their possible role in the mineralization of organic matter in a mesotrophic lake, Yuno-Ko. Jap. J. Ecol. 20, 151-158.

THORNLEY M.J. (1960) The differentiation of Pseudomonas from other Gram-negative bacteria on the basis of arginine metabolism. J. appl. Bact. 23, 37-52.

THORNLEY M.J. (1967) A taxonomic study of Acinetobacter and related genera. J. Gen. Microbiol. 49, 211-256.

TRUST T.R. (1972) The bacterial population in vertical flow tray hatcheries during incubation of salmonid eggs. J. Fish. Res. Bd. Canada 29, 567-571.

TSERNOGLOU D. and ANTHONY E.H. (1971) Particle size, water stable aggregates and bacterial populations in lake sediments. Can. J. Microbiol. 17, 217-227.

TUKEY H.B. Jr. (1971) Leaching of substances from plants. In Ecology of Leaf Surface Micro-organisms (T.F. Preece and C.H. Dickinson, eds.). Academic Press, London. 67-80.

VACCARO R.F. and JANNASCH H.W. (1966) Studies on heterotrophic activity in sea water based on glucose assimilation. Limnol. Oceanogr. 11, 596-607.

van SOESTBERGEN A.A. and CHING HO LEE (1969) Pour plates or streak plates? Appl. Microbiol. 18, 1092-1093.

WAID J.S., PRESTON K.J. and HARRIS P.J. (1971) A method to detect metabolically-active microorganisms in leaf litter habitats. Soil Biol. Biochem. 3, 235-241.

WAKSMAN S.A. (1941) Aquatic bacteria in relation to the cycle of organic matter in lakes. Symposium on Hydrobiology. Madison University Press, Wisconsin. 86-105.

WAKSMAN S.A., REUSZER H.W., CAREY C.L., HOTCHKISS M. and RENN C.E. (1933) Studies on the biology and chemistry of the Gulf of Maine. III. Bacteriological investigations of the sea water and marine bottoms. Biol. Bull. 64, 183-205.

WAKSMAN S.A., STOKES J.L. and BUTLER M.R. (1937) Relation of bacteria to diatoms in sea water. J. mar. biol. Ass. U.K. 22, 359-373.

WEEKS O.B. (1944) Populations of heterotrophic bacteria in two sediment layers of Western Lake Erie. J. Bact. 47, 446-447.

WEEKS O.W. (1969) Problems concerning the relationships of cytophagas and flavobacteria. J. appl. Bact. 32, 13-18.

WETZEL R.G. (1964) A comparative study of the primary productivity of higher aquatic plants, periphyton and phytoplankton in a large shallow lake. Int. Revue ges. Hydrobiol. Hydrogr. 49, 1-61.

WETZEL R.G. (1969) Excretion of dissolved organic compounds by aquatic macrophytes. Bioscience 19, 539-540.

WILLIAMS F.T. and McCOY E. (1935) The microflora of the mud deposits of Lake Mendota. J. sed. Petrol. 5, 31-36.

WILSON W.B. and COLLIER A. (1972) The production of surface-active material by marine phytoplankton cultures. J. mar. Res. 30, 15-26.

WINOGRADSKY S. (1929) Études sur la microbiologie du sol. Sur la dégradation de la cellulose dans le sol. Annls Inst. Pasteur, Paris 43, 549.

WOOD E.J.F. (1963) Heterotrophic microorganisms in the oceans. Oceanogr. mar. Biol. A. Rev. 1, 197-222.

WOODBRIDGE D.D. and GARRETT W.R. (1969) Relationship between bacteria, nutrients and rainfall in selected bodies of fresh water. Bull. env. Contam. Toxicol. 4, 311-319.

WOOLHOUSE H.W. (1967) The nature of senescence in plants. S.E.B. Symposium 21, 179-213.

WRIGHT R.T. (1970) Glycollic acid uptake by planktonic bacteria. Occ. Pap. Inst. mar. Sci. Alaska 1, 521-536.

WRIGHT R.T. and HOBBIE J.E. (1965) The uptake of organic solutes in lake water. Limnol. Oceanogr. 10, 22-28.

WRIGHT R.T. and HOBBIE J.E. (1966) Use of glucose and acetate by bacteria and algae in aquatic ecosystems. Ecology 47, 447-464.

WUHRMANN K. (1964) River bacteriology and the role of bacteria in self-purification of rivers. In Principles and Applications in Aquatic Microbiology (H. Heukelekian and N.C. Dondero, eds.). Wiley, New York. 167-192.

ZOBELL C.E. (1946) Marine Microbiology. Chronica Botanica, Waltham, Mass.

APPENDIX 1 Samples discussed in Chapter 3

Date sampled	Open water	Water over weed	Water over harbour spring
15/ 4/69	+ ch	+ ch	-
17/ 6/69	+ ch	+ ch	-
28/10/69	+ ch	+ ch	-
25/11/69	+ ch	-	+ ch
17/ 3/70	+ ch	+ ch	+ ch
20/ 4/70	+ ch	-	+ ch
19/ 5/70	+	+	+
21/ 6/70	+	+	+
27/ 7/70	+	+	+
20/ 8/70	+	+	+
9/ 9/70	+	+	+
9/10/70	+	+	+
5/11/70	+	+	+
7/12/70	+	+	+
5/ 1/71	+	+	+
1/ 2/71	+	+	+
24/ 2/71	+	+	+
7/ 4/71	+ dc	-	-
19/ 4/71	+ dc	-	-
13/ 7/71	+ ch dc	+ ch	-
5/12/71	+ dc	-	-

- no samples collected.

+ samples collected and bacteria counted by pour plate method.

ch bacteria characterized.

dc bacteria counted by direct microscopic method.

APPENDIX 2 Samples discussed in Chapter 4

Date sampled	Open water	Water over Elodea	Inlets H	A	C	E
28/10/69	+	-	-	-	-	-
25/11/69	+ ch	-	+ ch	+ ch	+ ch	+ ch
17/ 3/70	+ ch	+ ch	+ ch	-	-	-
20/ 4/70	+ ch	-	+ ch	-	-	-
19/ 5/70	+	+	+	-	-	-
21/ 6/70	+	+	+	-	-	-
27/ 7/70	+	+	+	-	-	-
20/ 8/70	+	+	+	-	-	-
9/ 9/70	+	+	+	-	-	-
9/10/70	+	+	+	-	-	-
5/11/70	+	+	+	-	-	-
7/12/70	+	+	+	-	-	-
5/ 1/71	+	+	+	-	-	-
1/ 2/71	+	+	+	-	-	-
24/ 2/71	+	+	+	-	+	AUT-
8/ 3/71	+	-	-	-	+	-
22/ 3/71	+ AUT	-	-	-	+	-

- no samples collected.
+ samples collected and bacteria estimated by the pour plate method.
ch 40-50 bacteria characterized.
AUT uptake of radioactive compounds by bacteria studied.

APPENDIX 3 Results of tests carried out on isolates of Enterobacteriaceae selected for further study

A Lactose fermented within 2 days at 37°C

date sampled	Isolate source	no.	MR	VP	citrate	urease	salicin	inositol	gelatin	H_2S	arginine	flagella	phenyl-alanine	Genus assigned to
15/ 4/69	Over weed	54	-	+	-	-	-	-	-	-	+	per	-	Enterobacter
15/ 4/69	Over weed	120	-	+	-	-	-	-	-	-	+	per	-	Enterobacter
15/ 4/69	Over weed	133	-	+	-	-	-	-	-	-	+	per	-	Enterobacter
15/ 4/69	Over weed	195	-[1]	+[1]	-	-	-	-	-	-	+	per	-	Enterobacter
15/ 4/69	Mud	369	+[1]	-[1]	-	-	-	-	-	-	NT	NE	-	Escherichia
17/ 6/69	Open water	20	-	+	+	-	-	-	-	-	+	per	-	Enterobacter
11/11/69	C	15	-	+	-	-	-	-	+	-	-	per	-	Enterobacter
25/11/69	Open water	38	+	-	+	-	+	+	-	-	-	per	-	Escherichia
25/11/69	E	1	+	-	+	+	-	-	+	-	+	NE	-	Proteus
25/11/69	E	5	+	-	+	-	+	-	-	-	-	per	-	Escherichia
25/11/69	E	48	+	-	+	-	+	-	-	-	-	per	-	Escherichia
17/ 3/70	Over weed	26	-	+	NT	NT	NT	NT	NT	NT	NT	per	NT	Enterobacter
20/ 4/70	Open water	3	-	+	NT	NT	NT	NT	NT	NT	NT	per	NT	Enterobacter
20/ 4/70	Open water	12	-	+	NT	NT	NT	NT	NT	NT	NT	per	NT	Enterobacter
20/ 4/70	Open water	22	-	+	NT	NT	NT	NT	NT	NT	NT	per	NT	Enterobacter
20/ 4/70	Open water	38	-	+	NT	NT	NT	NT	NT	NT	NT	per	NT	Enterobacter

[1] not retested after 4 days incubation; per - peritrichous flagella; per,sub - some peritrichous flagella but mainly one or two subpolar flagella; NT - not tested; NE - not examined.

B Lactose fermented, but not within 2 days at 37°C

15/ 4/69	Over weed	210	+	-	+	-	-	-	-	-	+	per	-	Salmonella
17/ 6/69	Over weed	52	-	+	+	-	+	-	NT	-	+	per	-	Enterobacter
17/ 6/69	Open water	4	+	+	+	-	-	+	+	-	-	per	-	Enterobacter
28/10/69	Over weed	3	-	+	+	-	-	-	-	-	+	per	-	Enterobacter
28/10/69	Elodea	2	+	-	-	-	-	-	-	-	-	per,sub	-	Salmonella
28/10/69	Elodea	11	+	-	-	-	-	-	-	-	-	per	-	Salmonella
28/10/69	Elodea	41	+	+	-	-	-	-	-	-	-	per,sub	-	Salmonella
17/ 3/70	Elodea	44	-	+	-	-	-	-	-	-	-	per	-	Enterobacter
17/ 3/70	Over weed	14	-	+	+	-	-	-	-	-	+	per	-	Enterobacter
20/ 4/70	Open water	2	+	-	+	-	-	-	-	-	+	per	-	Salmonella
20/ 4/70	Open water	23	-	+	+	+	-	-	-	-	+	per	-	Proteus
20/ 4/70	Open water	35	-	+	+	-	-	-	-	-	+	per	-	Enterobacter

APPENDIX 3 (cont.)

C Lactose not fermented at 30°C or 37°C after incubation for 4 days, or only fermented at 37°C after 4 days

date sampled	Isolate source	no.	MR	VP	citrate	urease	salicin	inositol	gelatin	H_2S	arginine	flagella	phenyl-alanine	Genus assigned to
15/ 4/69	Mud	410	-	+	+	-	-	-	-	-	NT	per	-	Serratia
11/11/69	C	1	+	+	+	+	-	+	-	+	-	per	-	Proteus
11/11/69	C	44	+	-	+	+	-	+	-	+	-	per	-	Proteus
11/11/69	Open water	38	+	-	-	-	-	-	-	-	-	NE	-	Salmonella
17/ 3/70	Open water	3	-	+	-	+	-	-	-	-	+	per	-	Proteus
17/ 3/70	Over weed	20	-	+	+	+	+	-	-	-	+	per	-	Proteus
17/ 3/70	Over spring H	47	-	+	-	-	-	-	-	-	-	per	-	Enterobacter
7/12/70	Open water	25	-	+	-	-	-	-	NT	-	-	pol	-	Vibrio
13/ 7/71	Elodea	50	+[1]	+[1]	+	-	-	-	+	+	+	pol	-	Aeromonas
13/ 7/71	Mud	3	+[1]	+[1]	+	-	-	-	+	+	+	NE	-	Aeromonas

1 These two isolates were not retested after 4 days incubation.

APPENDIX 4 Samples discussed in Chapter 6

Date sampled	*Elodea canadensis*	Water over weed	Open water
15/ 4/69	-	+ ch	+ ch
17/ 6/69	-	+ ch	+ ch
28/10/69	+ ch[1]	+ ch[1]	+ ch
17/ 3/70	+ ch	+ ch	+ ch
20/ 4/70	-	-	+
19/ 5/70	+	+	+
21/ 6/70	+	+	+
27/ 7/70	+	+	+
20/ 8/70	+	+	+
9/ 9/70	+	+	+
9/10/70	+	+	+
5/11/70	+	+	+
7/12/70	+	-	+
5/ 1/71	+	+	+
1/ 2/71	+	+	+
24/ 2/71	-	+	+
13/ 7/71	+ ch dc	+ ch	+ ch dc

- \- no samples collected.
- \+ samples collected and bacteria estimated by pour plate method.
- ch bacteria characterized.
- dc bacteria estimated by direct microscopic method.

[1] Weed growing in both 2 m and 6 m of water, and water over these two depths of weed, were sampled.

APPENDIX 5 Sampling dates of results presented in Fig. 7.3

Date sampled	Organisms estimated (open water samples from 3 m)
2/ 9/70	z
9/ 9/70	b z
9/10/70	b
12/10/70	z
27/10/70	z
5/11/70	b
9/11/70	b p z
16/11/70	b p z
23/11/70	b p z
7/12/70	b p z
14/12/70	z
5/ 1/71	b p z
15/ 1/71	z
1/ 2/71	b p z
11/ 2/71	z
24/ 2/71	b p z
8/ 3/71	b p z
22/ 3/71	b p z
7/ 4/71	b p z
19/ 4/71	b p z
3/ 5/71	b p z
19/ 5/71	z
9/ 6/71	b p z
23/ 6/71	z
13/ 7/71	b p z

b - bacteria; p - phytoplankton; z - zooplankton.

APPENDIX 6 Dimensions of dominant algae

Alga	Date sampled	Dimensions in μm (Mean and standard deviation)				No. cells in unbroken filaments
		length	width (girdle view)	width (valve view)	diameter	
	9/11/70	94 ± 3 (10)	2.4 ± 0.2 (5)	2.3 ± 0.2 (5)		
	7/12/70	100 ± 3 (10)	2.2 ± 0.4 (5)	2.3 ± 0.2 (5)		
	7/ 4/71	94 ± 10 (10)	2.3 (1)	2.3 (1)		
Diatoma elongatum	19/ 4/71	97 ± 3 (10)	2.5 ± 0.4 (5)	2.1 ± 0.2 (5)		
	19/ 5/71	92 ± 3 (4)	2.7 ± 0.1 (3)	2.3 (1)		
	9/11/70	58 ± 6 (10)	2.7 ± 0.9 (8)	2.5 ± 0.3 (2)		
	5/ 1/71	53 ± 4 (9)	2.5 ± 0.6 (9)	-		
Asterionella formosa	3/ 5/71	56 ± 5 (3)	3.2 (1)	-		
	19/ 5/71	53 ± 3 (2)	3.4 ± 0.8 (2)	-		
	5/ 1/71	31 ± 8 (10)			4.6 ± 0.2 (10)	-
Melosira granulata var. angustissima	1/ 2/71	25 ± 4 (10)			4.5 ± 0.6 (10)	14 ± 1 (3)
	24/ 2/71	24 ± 2 (10)			4.5 ± 0.5 (10)	16 ± 3 (5)
	19/ 4/71	32 ± 2 (4)			4.3 ± 0.3 (4)	-
	16/11/70	2.7 ± 0.5 (10)			6.7 ± 0.8 (10)	
	1/ 2/71	-			5.9 ± 0.5 (10)	
Cyclotella kützingiana	24/ 2/71	-			6.0 ± 1.0 (10)	
	19/ 4/71	-			5.1 ± 1.2 (10)	
	3/ 5/71	-			4.6 ± 0.3 (10)	
	19/ 5/71	-			5.3 ± 1.1 (10)	

numbers in brackets are numbers of cells measured

APPENDIX 7 Seasonal variation in numbers of algae in the open water

Date sampled	No. cells per ml											
	Diatoma elongatum (Lyngb.) Agardh	*Asterionella formosa* Hass.	*Melosira granulata* (Ehr.) Ralfs var *angustissima* Müll.	*Cyclotella kützingiana* Thwaites	other diatoms including *Fragilaria* sp. *Synedra* sp. *Cocconeis* sp. *Cymbella* sp.	*Mougeotia* sp. ster.	*Cryptomonas* sp.	*Pandorina morum* (Müll.) Bory and *Eudorina elegans* Ehrnb[1]	small green zoospore or flagellate	other algae including *Oocystis* sp.[1]	*Dinobryon* sp.	Total dominant diatoms[2]	Total others
9/11/70	5160	515	0	170	30	0	10	0	0	0		5845	40
16/11/70	2810	210	0	150	40	0	55	0	0	0		3170	95
23/11/70	3125	100	0	105	55	0	80	0	480	0		3330	615
7/12/70	1460	305	0	90	90	0	0	0	0	0		1855	90
5/ 1/71	1065	515	3370	680	385	50	70	330	0	50		5630	885
1/ 2/71	410	265	4175	5870	90	0	0	70	0	25		10 720	185
24/ 2/71	0	20	1040	780	45	0	0	30	0	0		1840	75
8/ 3/71	0	30	830	465	55	0	0	0	0	0		1325	55
22/ 3/71	0	0	145	1290	100	0	0	50	0	0		1435	150
7/ 4/71	975	0	20	3270	70	0	0	230	0	0		4265	300
19/ 4/71	4030	0	155	1870	90	0	0	20	0	0		6055	110
3/ 5/71	1150	80	40	500	0	0	0	15	110	0		1770	125
19/ 5/71	460	120	45	310	15	0	0	0	835	0		935	850
9/ 6/71	215	265	40	1060	30	0	75	10	235	0		1580	350
13/ 7/71	425	245	0	340	25	0	55	0	150	0		1010	230
21/12/71	35	960	0	110	30	0	45	0	155	55		1105	285

1 individual cells of *Pandorina*, *Eudorina* and *Oocystis* were counted.

2 dominant diatoms are *Diatoma*, *Asterionella*, *Melosira* and *Cyclotella*.

APPENDIX 8 Samples discussed in Chapter 8

Date sampled	Sample: Open water	Littoral water	Littoral mud	Mud from beneath open water
9/11/70	d			
16/11/70	d			
23/11/70	d			
7/12/70	d			
5/ 1/71	d			
1/ 2/71	d			
24/ 2/71	d			
8/ 3/71	d			
22/ 3/71	d AUT			
7/ 4/71	d			d
13/ 7/71				d
5/12/71		d CUL		
21/12/71	d CUL	d CUL	CAU	
12/ 1/72	d AUT			
26/ 1/72		d	CAU	
8/ 2/72		d	d	
6/ 3/72		d		
27/ 3/72	d			

d - samples examined directly with phase-contrast illumination.

AUT - uptake of radioactive compounds by epiphytic bacteria studied.

CUL - samples used as inocula in attempts to culture epiphytic bacteria.

CAU - samples used as inocula for Caulobacter enrichments.

APPENDIX 9 Samples discussed in Chapter 9

Date sampled	Organisms estimated	
	Open water	Water over weed
15/ 4/69		b, ch mb, ch
17/ 6/69		b, ch mb, ch
19/ 5/70	b mb z	
3/ 6/70	z	
21/ 6/70	b mb z	
7/ 7/70	z	
27/ 7/70	b mb z	
6/ 8/70	z	
20/ 8/70	b mb	
2/ 9/70	z	
9/ 9/70	b mb z	
9/10/70	b mb	
12/10/70	z	
27/10/70	z	
5/11/70	b mb	
9/11/70	b z p	
16/11/70	b z p	
23/11/70	b z p	
7/12/70	b mb z p	
14/12/70	z	
5/ 1/71	b mb z p	
15/ 1/71	z	
1/ 2/71	b mb z p	
11/ 2/71	z	
24/ 2/71	b z p	
8/ 3/71	b mb z p	
22/ 3/71	b mb z p	
7/ 4/71	mb and smb	
13/ 7/71	b, ch smb, ch	

b - bacteria; ch - bacteria characterized; z - zooplankton p - phytoplankton; mb - bacteria sampled from mud beneath water; smb - bacteria in surface layer of mud sampled.

APPENDIX 10 Experimental data on cytophagas expressed in form used in Adansonian analysis

Isolate	Features included in analysis of Colwell (1969)																																				
	Length (μm)			Helical	Slight coiling	Pigment (Type-Lewin and Lounsbery)							Agar digested	Alginate digested	Methyl cellulose or carboxy-methyl cellulose digested	Starch hydrolyzed	Catalase present	Oxidase present	Tyrosine			H_2S produced	Penicillin conc. permitting growth[1]			Sodium lauryl sulphate sensitivity	Salinity tolerance[2]				Temperature permitting growth (°C)[1]		
	< 10	10-50	> 50			I	II	III	IV	IVa[1]	V	VI							Growth	Clearing	Ring		100 units/disc (~ 0.6 g/l)	10 units/disc (~ 0.06 g/l)	0.000001 g/l		fresh	0.5 S	1 S	2 S	30	35	40
1ST 191	+	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	-	-	+	+	+	+	-	+	+	-	-	+	-	-
5H 2	+	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	-	-
5H 4	+	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	-	+	-	-
12W 1	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	+	+	-	+	+	-	+	+	+	+	+	-	-	+	-	-
12W 2	+	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+	+	+	+	-	+	+	+	+	+	-	+	+	-	-	+	-	-
13D3 1	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	+	+	-	-	+	-	+	+	-	+	+	-	-	+	-	-
13D3 2	+	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	-	-	+	-	+	+	-	+	+	-	-	+	-	-
13P 1	+	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	+	-	+	-	-
13P 2	+	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	+	-	+	-	-
C. lytica LIM-21	-	+	-	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	-

[1] feature modified or additional features included.

[2] S - concentration of NaCl equivalent to salinity of sea water.

APPENDIX 10

(cont.)

Isolate	Features included in analysis of Colwell																		Tests carried out by Lewin and Lounsbery (1969) but not included in analysis of Colwell									
	Nitrogen source				Carbon source														Temperature tolerance (°C)		Gelatin liquefaction	Filter paper digested	Litmus milk reaction				Nitrate reduction	
	Tryptone	Casamino acids	Na glutamate	Na nitrate	Na acetate (5 g/l)	Na acetate (1 g/l)	Na lactate (5 g/l)	Na lactate (1 g/l)	Glycerol (5 g/l)	Glycerol (1 g/l)	Glucose (5 g/l)	Glucose (1 g/l)	Galactose (5 g/l)	Galactose (1 g/l)	Sucrose (5 g/l)	Sucrose (1 g/l)			+ 3	- 196			Acid	Reduction	Clotting	Proteolysis		
1ST 191	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			+	+	+	-	+	-	-	-	-	
5H 2	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-			+	+	+	-	+	-	-	-	+	
5H 4	+	+	+	-	+	+	-	+	+	+	+	+	+	+	-	-			+	+	+	-	+	-	-	-	+	
12W 1	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+			+	+	+	-	+	-	-	-	+	
12W 2	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-			+	+	+	-	+	-	-	-	+	
13D3 1	+	+	+	-	+	+	+	+	+	-	+	+	+	+	-	-			+	+	+	-	+	-	-	-	+	
13D3 2	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+			+	+	+	-	-	-	-	-	+	
13P 1	+	+	+	-	+	+	+	+	+	-	+	+	+	+	-	-			+	+	+	-	+	-	-	-	+	
13P 2	+	+	+	-	-	+	+	+	+	+	+	+	+	+	-	-			+	+	+	-	+	-	-	-	+	
C. lytica LIM-21	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+			+	+	+	-	-	+	+	-	-	